A study of P13K regulation by costimulatory and inhibitory receptors in T and B lymphocytes

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Award date:
2000

Awarding institution:
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Dedicated to the memory of Daisy Shaw.

A STUDY OF PI3K REGULATION BY COSTIMULATORY AND INHIBITORY RECEPTORS IN T AND B LYMPHOCYTES

submitted by

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For the degree of PhD
of the University of Bath
2000

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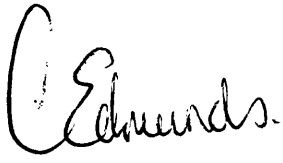

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ABSTRACT

PI3K has been described as a key regulator of signalling cascades in T and B lymphocytes. Particularly, PI3K has been proposed as an essential component of the CD28 mediated costimulatory signal which, in the presence of antigen specific signals delivered by the TCR, drives IL-2 production, T cell proliferation and survival. However, as PI3K deficient mice display impaired B cell responses with no T cell signalling defects, some reports have questioned the significance of PI3K in CD28 mediated T cell activation.

This study presents novel data which further examines the relationship between PI3K and CD28: A further level of PI3K regulation is mediated by CD28 via the differential recruitment of p110δ and p110β PI3K catalytic subunits. CD28 activates the unique serine kinase capacity of p110δ that results in the down regulation of p110δ lipid kinase activity. Furthermore the CD28 mediated tyrosine phosphorylation of SHIP, which catalyses the degradation of PI(3,4,5)P_3, is described. Evidence is presented which demonstrates that basally high levels of 3'-phosphoinositides exist in the Jurkat T cell line that are not subject to regulation by lipid phosphatases. Evidence presented here may explain previous data that disputed the role of CD28 mediated PI3K activation in Jurkats.

This study has also investigated the mechanisms by which PI3K is regulated in B cells: In addition to the enzymatic activity of SHIP, PI3K activity in B cells may be negatively regulated via sequestration of p85 by the adaptor protein Gab2. In addition this study presents the first report of a potential role for SHP2 in coupling the BCR to MAPK activation, and indicates that the SHP2 PTPase mediated release of SHP2 from the Gab2/PI3K complex may play a role in inhibitory signalling. The examination of Gab2 and SHP2's relationship to PI3K in CD28 stimulated T cells would be of future interest.

ACKNOWLEDGEMENTS

I would, firstly like to acknowledge the Medical Research Council for funding this study. Secondly, I must acknowledge my supervisor Stephen Ward, for giving me the opportunity to undertake this research, and for directing my work during the last three years. Other Wardoids past and present should also be spoken of here: Yannis, it was an interesting pleasure, and Richard, thanks for the POR principal (Don't check the abbreviations section, its not there!). The current Wardists should not go unmentioned, Marisa, Budgie, and Gill (and ex- Wardist Jane, wherever you are) and honorary members (Helen), cheers for the laughs and organised coffee breaks, its been emotional!

I would also like to thank the Welham group for their help, advice and reagents, and the labs of Bart Vanhaesebroeck, Carl June, Chris Rudd, and Mark Coggeshall, for antibodies and cell lines.

On a more personal note I especially want to say thankyou to Mr and Mrs Raven, and of course their more senior offspring Maria: I am grateful for the support you have always given me, and for your humour which is a continual source of inspiration! Also, and probably unconventionally, I would like to thank my in-laws who have 'been there' on countless occasions for Nick and I, and have made me feel welcome in their family. Other people who have made me laugh in the past three years (!) should also have their input to this thesis acknowledged, Andy-Fat man trapped in a thin mans body-Collin, and the Bentley's especially!!

Finally, but most importantly, I thank my husband Nick (what about me?!!), whom I would never have met had I not 'done' this PhD. I can't express enough appreciation for you being in my life, you're my best friend and I love you. For better for worse, team Edmunds!!!!!

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ABBREVIATIONS

ARF	adenosine ribosylation factor
BCR	B cell receptor
Ca2+	calcium
CD	cluster of differentiation
CD28RE	CD28 response element
CTLA4	cytotoxic T lymphocyte associated antigen 4
DAG	di-acyl glycerol
GAB2	Grb 2 associated binding protein 2
GEFs	guanine nucleotide exchange factor
Grb 2	growth factor receptor binding protein 2
ICOS	Inducible costimulator
IKK	IkB kinase
IRS-1	Insulin receptor substrate -1
ITAM	Immunoglobulin tyrosine based activation motif
ITIM	Immunoglobulin tyrosine based inhibition motif
ITK	Inducible T cell kinase
JNK	C-Jun N terminal kinase
kDa	kilodalton
LAT	linker for activated T cells
LY249002	2-(4-morpholinyl)-8-phenyl-4H-1-benzyopyran-4-one
mAb	Monoclonal antibody
mIg	Membrane bound immunoglobulin
NFkB	nuclear factor k B
PH	pleckstrin homology
PIF	PDK-1 Interacting fragment
PKB	protein kinase B

PKC	protein kinase C
PLCγ	phospholipase C gamma
PRK-2	PKC related kinase 2
PTEN	phosphatase and tensin homologue ten
PTK	protein tyrosine kinase
Ras GAP	Ras-GTPase activating protein
SEM	standard error of the mean
SH3	src homology 3 domain
SHIP	SH2 containing inositol polyphosphatase
SHP2	SH2 containing protein tyrosine phosphatase 2
SOC	store operated calcium channel
SOS	son of sevenless
Th	T helper
ZAP	zeta associated protein

AMINO ACID SINGLE LETTER CODE

A	Ala
C	Cys
D	Asp
E	Glu
F	Phe
G	Gly
H	His
I	Ile
K	Lys
L	Leu
M	Met
N	Asn
P	Pro
Q	Gln

R	Arg
S	Ser
T	Thr
V	Val
W	Trp
Y	Tyr

1 - INTRODUCTION

The fundamental aim of this study was to investigate the mechanisms which regulate phosphatidylinositol 3'- kinase (PI3K) activation within the context of T and B lymphocyte intra-cellular signalling pathways. In this section I shall firstly introduce the extensive family of PI3Ks and describe the biochemistry and regulation of these proteins. Secondly, I shall discuss the role of T and B lymphocytes within the immune system. I shall describe their cell surface receptors and intracellular proteins, and then summarise the biochemical pathways by which these proteins regulate lymphocyte activation.

1.1 PHOSPHATIDYINOSITOL 3'-KINASE

The PI3K family comprises a group of ubiquitously expressed proteins which were primarily identified through their ability to phosphorylate the D3-OH position on the inositol ring of phosphoinositide lipids (Stephens *et al* 1991). This lipid kinase activity regulates intracellular levels of 3'-phosphoinositides, which act as signal transducing second messengers (Arcaro *et al* 1993). The first PI3Ks to be purified and cloned were heterodimers of what is now considered the class 1A subfamily, consisting of a p110 catalytic subunit and a regulatory 85 kDa subunit (Otsu *et al* 1991). Different PI3K family members are now classified on the basis of their structural features, preferred lipid substrates, and coupled adaptor proteins. They are described in outline below in table 1 and in the following text:

1.1.1 PHOSPHATIDYLINOSITOL 3'-KINASE CLASSIFICATION

CLASS I

Class I PI3Ks utilise PI, PI(4) *P* and PI(4,5)P_2 as lipid substrates *in vitro*, whilst *in vivo*, the latter acts as the preferred substrate (reviewed by Vanhaesebroeck et al 1997a, Vanhaesebroeck et al 1999) . All Class I PI3Ks can coassociate with GTP bound Ras. The PI3Ks of class I occur as heterodimers comprising an enzymatic domain and an adaptor domain, the characteristics of which further divide the class into sub families: Class 1A PI3Ks comprise a 110 kDa catalytic domain (p110), within which the Ras binding domain, lipid kinase domain and carboxy terminal protein kinase domain are featured. p110 associates with an SH2 containing adaptor protein of 85 kDa, through which the catalytic domain is coupled to phosphotyrosine regulated signalling pathways. The unique phosphotyrosine containing motif with which the SH2 domains of the p85 regulatory subunit preferentially associate is YXXM. The presence of this sequence within the intracytoplasmic tail of cell surface receptors and adaptor proteins, has indicated the involvement of this class of PI3Ks in multiple signal transduction pathways. The p85

subunit also contains an SH3 region, which binds proline rich motifs, a proline rich region and a break point cluster (BCR) homology domain (BH) of unknown function. Multiple isoforms of the class 1A family of PI3K exist, namely, p85α, p85β and p110α, β and δ.

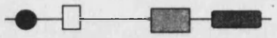
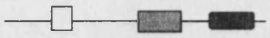
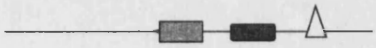
Class	Substrates	Subunits: Catalytic	Adaptor	Regulators	Schematic Structure (Catalytic Subunit)
IA	PtdIns, PtdIns(4)P_1 PtdIns(4,5)P_2	p110α,β,δ(m) Dp110(Dm) PIK1,PIK2 (Dd)	p85α,β(m) p55α, p50α(m) p60(Dm)	Tyr kinases Ras	
IB	PtdIns, PtdIns(4)P_1 PtdIns(4,5)P_2	p110γ(m) PIK3(Dd)	p101(m)	G-proteins (Bγ) Ras	
II	PtdIns PtdIns(4)P_1	PI-3 K C2α(m) PI-3 K C2β(m) PI-3 K 68D(Dm) PI-3 K C2 (Ce)	?	?	
III	PtdIns	$Vps34p^c$	p150(m) Vps15p(Sc)	Constitutive	

Table 1: Classification of PI-3 K Family members

Key: (Structural motifs) Black circle =adaptor binding region, white box=Ras binding domain, grey box=PIK homology domain, patterned oval=kinase domain white box= C2 domain (species) m=mammalian, Ce= Caenorhabditis elegans, Dm=Drosophila melanogaster, Dd=Dicosteleum discoideum, Sc=Saccharomyces cerevisiae.

The p110 isoforms of class 1A all share the class I PI3Ks characteristic broad substrate specificity and the ability to interact with Ras. P110δ is unusual in that it displays leukocyte restricted tissue distribution (Chantry *et al* 1997 and Vanhaesebroeck *et al* 1997b).

Class 1A PI3Ks also possess protein kinase activity. P110α has been demonstrated to phosphorylate a serine residue in the inter-SH2 domain region of p85α and p85β. In contrast p110δ harbours intrinsic autophosphorylation capacity so that it is phosphorylated on ^{1039} Ser. This autophosphorylation event is associated with a negative regulation of the lipid kinase activity (Vanhaesebroeck *et al* 1997b). Other structural features unique to p110δ include a leucine zipper and a proline rich region.

Class IB PI3Ks associate via larger adaptor proteins to G protein coupled receptors and are stimulated by G protein β and γ subunits. To date a 101 kDa adaptor has been identified. Additional reports have described that p110γ is responsive to activation via Gα

subunits (Murga *et al* 1998). Recently the activation of class 1A PI3Ks by heterodimeric G proteins has been described (Kowalski *et al* 1996), and, although Gβγ activation of class 1A catalytic subunits may occur via Gβγ upstream activation of PI3Ks. It appears that Gα may directly activate p110β (Roche *et al* 1998). Knockout mice of various class I PI3Ks have been made and the phenotypic effects of these are summarised in table 2.

CLASS II

The Class II PI3Ks preferentially phosphorylate PI(4) *P* and are incapable of phosphorylating PI(4,5)P_2 *in vitro*. These PI3Ks are predominantly membrane located in contrast to class I and class II PI3Ks which are cytoplasmically located. Class II PI3Ks contain a C2 domain at their carboxyl terminus, which is lacking critical Asp residues that are usually needed within this structural motif for Ca^{2+} binding. However this class of PI3Ks exhibit Ca^{2+} independent anionic lipid binding properties. Pathways to which these PI3Ks couple have yet to be identified.

TARGET	PHENOTYPE	INTERPRETATION
P85α (partial disruption)	Altered insulin responsiveness Severe B cell immunodeficiency	P50α and p55α still made. Phenotypes resemble BTK K/O (Terauchi *et al* 1999)
P85α (complete disruption)	Neonatal lethal In Rag 2 chimaeras: B cell maturation Impaired, No T cell defects	Cause of lethality not established. (Suzuki *et al* 1999, Fruhman et 1999)
P85β (complete disruption	No apparent defects.	Unclear (Not-published)
P110α	Embryonic lethal	Complex changes in PI3K expression and function (Bi *et al* 1999).
P110γ (complete disruption)	Impaired neutrophil and macrophage function. Impaired T cell survival and function.	Neutrophils and macrophages display impaired migration by chemotaxis, impairment respiratory burst in macrophages (Condliffe *et al* 1999). Impaired T cell survival and $CD8^+$ responses, (NB. Un-published data (M. Wymann) brings into question initial observations of this phenotype)

Table 2: Characteristics of PI3K knockout mice.

CLASS III

Class III PI3Ks can be characterised by their restricted substrate specificity as they only catalyse the phosphorylation of PtdIns, producing PI(3)*P*. They seem to be involved in housekeeping roles including membrane trafficking and vesicle morphogenesis, and have a functional and structural homologue in the yeast PI3K, Vps34p (Volinia *et al* 1995).

1.1.2 MEASUREMENT OF PI3K ACTIVITY

The development of methodologies by which PI3K activity can be measured has aided research into the pathways leading to PI3K biochemical activation. A widely used technique has been applied by many studies (Hawkins *et al* 1992, Ward *et al* 1993) which involves the labelling of cells with [^{32}P]-orthophosphoric acid. Labelled phosphatidylinositol lipids can be separated via HPLC by an ammonium phosphate gradient, and determination of the accumulation of distinct lipids species can be made. A further technique was developed which measured the accumulation of PI(3,4,5)P_3 via the use of an alkali enzyme treatment which releases IP(1,3,4,5)P_4 from PI(3,4,5)P_3. The accumulated IP(1,3,4,5)P_4 which can then be measured by an IP(1,3,4,5)P_4 binding assay (Van der Kaay *et al* 1997). Techniques which allow the measurement of intracellular changes in cellular levels of 3'-phosphoinositide lipids have been developed, which employ green fluorescent protein tagged pleckstrin homology domains. The PKB PH domain has been exploited in this way to monitor PI(3,4)P_2 and PI(3,4,5)P_3 accumulation (Franke *et al* 1997, Watton *et al* 1999). Pleckstrin homology domains will be discussed later in this Introduction. Finally the use of antibodies which recognise the phosphorylated form of the PI3K effector molecule PKB are becoming a more widespread tool for the detection of PI3K activation in biological systems (Astoul *et al* 1999)

1.1.3 REGULATION OF CLASS 1A PI3Ks

This study will concentrate on the class 1A PI3K family members and use of the term PI3K should be taken to mean class 1A p85/p110 heterodimeric PI3K from hereon in this study. Recent models for PI3K activation describe the binding of the p85 regulatory subunit to adaptor proteins which recruit the enzyme to the plasma membrane where it is activated (Reif *et al* 1996). This model was proposed following the observation that constitutive membrane targeting of the p110 catalytic subunits of PI3K creates a constitutively active enzyme that generates PI(3,4,5)P_3 and PI(3,4)P_2 when expressed in cells (Reif *et al* 1996).

Upon membrane localisation, upregulation of class 1A PI3Ks may occur via direct association with Ras-GTP. Reports have shown that Ras can activate class 1A PI3Ks *in vitro,* in a GTP dependent manner (Rodriguez Viciana *et al* 1996), and *in vivo*, co-expression of PI3K p110α-p85α heterodimers, with active mutants of Ras, has been shown to upregulate P110 catalytic activity (Rodriguez-viciana *et al* 1994, Marte *et al* 1997). Interestingly, data also exists which places Ras downstream of PI3K (Kurz *et al* 2000). Additionally, in some cell types, regulation of p85 and p110 by serine / threonine phosphorylation has been observed (Reif *et al* 1993), and reports that $p59^{fyn}$ PTK can act as an activator of PI3K exist (Pleiman *et al* 1994). $P59^{fyn}$ has been demonstrated to interact with p85, via an association between the SH3 domains of $p59^{fyn}$ and a proline rich motif within the p85 subunit, which leads to tyrosine phosphorylation of p85 at Y^{688} and correlates with enhanced PI3K activity (Pleiman *et al* 1994).

1.1.4 NEGATIVE REGULATION OF PI3K DEPENDENT PATHWAYS

The lipid products of PI3K are not hydrolysed by PLC-γ1, and as $PI(3,4,5)P_3$ has been demonstrated to be a potent activator of downstream signalling proteins (Toker *et al* 1997), and many PH domain containing molecules have been demonstrated to bind $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (Lietzke *et al* 2000), the regulation of intracellular levels of these lipids by inositol phosphatases is of major interest. Consequently much research has focused on the physiological and biochemical roles of the inositol polyphosphatases SHIP and PTEN, which have been implicated as major regulators of PI3K lipid product accumulation.

SHIP

The modulation of PI3K signalling pathways by SHIP is the focus of much of the work

GROUP	SUBSTRATES	INOSITOL-5 PHOSPHATASE	ROLE
TYPE I	$Ins(1,4,5)P_3$ $Ins(1,3,4,5)P_4$	e.g. platelet 5'phosphatase enzyme	Ca^{2+} regulation (Matzaris *et al* 1994)
TYPE II	$Ins(1,4,5)P_{3+}$, $Ins(1,3,4,5)P_4$, $PI(4,5)P_2$ $PI(3,4,5)_3$	OCRL-1, Synaptojanin, Synaptojanin II.	Lysosome trafficking, vesicle trafficking. (Ross *et al* 1991, McPherson *et al* 1996,)
TYPE III	$Ins(1,3,4,5)P_4$ $PI(3,4,5)P_3$	SHIP and SHIP II	EGFR, PDGFR, TCR, FCγRIIB (Damen *et al* 1996, Pessesse *et al* 1998)

Table 3: Inositol polyphosphate 5'phosphatase family

described in this study. SHIP is a 145 kDa SH2 containing type III member of the inositol polyphosphate 5' phosphatase family (See table 3), which include enzymes that selectively de-phosphorylate the 5' position of the inositol ring on 5' phosphorylated phosphoinositide- and inositol- lipids (Drayer *et al* 1996). Members of this family are defined by two signature motifs: (F/I)WXGDXN (F/Y)R and (R/N)XP(S/A)(W/Y)(C/T)DR(I/V)(L/I) (Majerus *et al* 1999).

SHIP has a unique substrate specificity for lipids phosphorylated at the 3' position of the inositol ring, namely Inositol 1,3,4,5-tetra-kis phosphate (Ins(1,3,4,5)P_4), and PI(3,4,5)P_3, the D-3 phosphoinositide PI3K metabolic product (Damen *et al* 1996). Removal of the 5' phosphatase from these lipids produces Ins(1,3,4) tris phosphate (Ins(1,3,4)P_3) and PI(3,4)P_2 which results in the down regulation of the effects of PI3K on mitogenesis, cellular transformation and immune cell function (Chacko *et al* 1997, Ono *et al* 1997, Tridandapani *et al* 1997b).

Structurally, SHIP possesses an N-terminal SH2 domain, a phosphatase domain, bearing the phosphatase core consensus motifs, two NPxY concensus sequences that are targeted

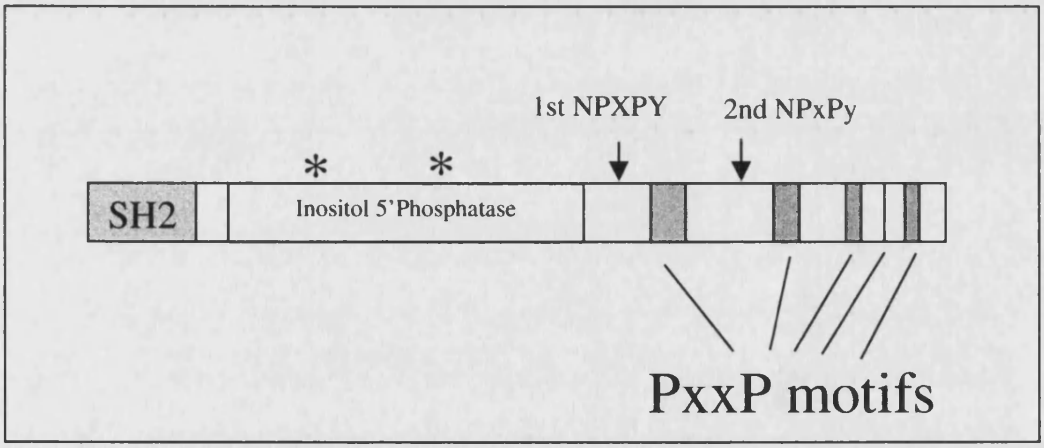


Diagram 1: Modular structure of 145 kDa SHIP

SHIP comprises an SH2 domain which mediates protein - protein interaction with tyrosine phosphorylated proteins. Asterisks above the phosphatase domain mark regions of homology shared all 5' - phosphatases. The C terminal NPXPY motifs, when phosphorylated have phosphotyrosine binding domain (PTB) binding potential. Also depicted above are several PXXP, poly- proline rich, motifs, three of which have good homology for SH3 domain binding (Thicker grey boxes), whilst the others show weaker SH3 binding homology, represented by thinner bands.

for phosphotyrosine binding (PTB) domains and several proline rich domains (Damen *et al* 1996, Rohrschneider *et al* 2000) (See Diagram 1). The minimum catalytic core of SHIP has recently been identified to lies between amino acids 400-866 (Aman *et al* 2000). The SH2 domain of SHIP has specificity for the phosphotyrosine recognition sequence YSNL

which is present within the FCγRIIB in B cells (D'Ambrosio *et al* 1995), and c-kit in haemopoietic cells (Yarden *et al* 1987).

SHIP is subject to phosphorylation in a variety of haemopoietic cell lines and lymphocytes. Phosphorylation of SHIP has been described to occur in haemopoietic cell lines in response to cytokine receptor stimulation with IL-2, IL-3 (Liu *et al* 1997b) and IL-4 (Giallourakis *et al* 2000). Growth factor receptor stimulation, following treatment of cells with erythropoietin (Damen *et al* 1993, Verdier *et al* 1997), Insulin, EGF, PDGF, IGF-1 and NGF (Habib *et al* 1998), have also been reported to mediate the tyrosine phosphorylation of SHIP. Studies in T and B lymphocytes have shown that SHIP is phosphorylated following TCR engagement and IL-4 stimulation (Lamkin *et al* 1996), and ligation of the FCγRIIB was demonstrated to mediate SHIP phosphorylation in B lymphocytes and mast cells (Chacko *et al* 1996). Various studies have sought to identify the PTKS which phosphorylate SHIP. In T cells, recombinant Lck has been demonstrated to phosphorylate SHIP (Lamkin *et al* 1997). Meanwhile studies, studies using PTK deficient B cell lines suggest that Lyn may be more important for the tyrosine phosphorylation of SHIP than Syk in B lymphocytes (Nagai *et al* 1995), and more recent work further supports a role for Lyn as the predominant mediator of SHIP phosphorylation in B lymphocytes (Phee *et al* 2000).

Conflicting evidence exists as to the importance of tyrosine phosphorylation in the catalytic activation of SHIP. In platelets tyrosine phosphorylation of SHIP following thrombin stimulation does not correlate with its up-regulation, but is correlated with its relocalisation to the actin cytoskeleton (Giuriato *et al* 1997). Thus SHIP may not be responsible for the observed accumulation of PI(3,4)P_2 upon thrombin stimulation and aggregation of platelets. However previous studies by this group have observed that tyrosine phosphorylation and enzymatic degradation of Ins(1,3,4,5)P_4 are closely correlated in T lymphocytes (Edmunds *et al* 1999). Moreover, recent studies have identified key C-terminal tyrosine residues which appear to be essential for SHIP's enzymatic activity (Aman *et al* 2000). These data, along with those described by another study (Phee *et al* 2000) imply that the enzymatic activation of SHIP may be mediated via membrane localisation, which is directed by phosphorylation of this C-terminal motif.

SHIP has been implicated in negative signalling pathways initiated by IL-3 and GMCSF (Liu *et al* 1999), and in B cells following co-ligation of the BCR and FCγRIIB (Liu *et al* 1998), via its enzymatic degradation of PI(3,4,5)P_3. Moreover, through its non catalytic domains, SHIP has been proposed to mediate inhibitory signalling independently of its

enzymatic function. SHIP is implicated in the inhibition of Ras activation, through the sequestration of Shc, via an association between the SH2 domain of SHIP and tyrosine phosphorylated Shc (Liu *et al* 1997). A further interaction between these two proteins occurs through the SHIP NPxPY and the PTB binding domain of Shc, which may serve to stabilise the interaction between Shc and SHIP (Harmer *et al* 1999).

Further evidence as to SHIP's functional role has been gained from the analysis of SHIP knockout mice. Whilst these mice are viable and fertile, homozygotes have a reduced lifespan which is due to myeloid cell infiltration of vital organs (Liu *et al* 1999). Haemopoietic lineages from these mice display increased colony formation in response to IL-3 GM-CSF and G-CSF, which may reflect a role for SHIP in regulating either survival or proliferation in response to these cytokines. SHIP-/- myeloid and B cells also exhibit elevated resting levels of PI(3,4,5)P_3, which correlate with increased PKB activity, and are sensitive to the PI3K inhibitor wortmannin (Helgason *et al* 1998). SHIP null animals show a hyperplasia of macrophage and granulocyte lineages, at the expense of B cell production. The reduced pool of SHIP deficient B cells demonstrate a marked elevation of resting calcium flux which is further elevated above wild type levels upon stimulation, and correlates with increased proliferation *in vitro* (Liu 1998).

PTEN

The human tumour suppressor gene phosphatase and tensin homologue 10 (*PTEN*) encodes a dual specificity serine/threonine and tyrosine phosphatase which has been demonstrated to de-phosphorylate a limited array of acidic substrates eg: poly-Glu-phospho-Tyr (Myers *et al* 1997). PTEN has since been demonstrated to dephosphorylate 3'-phosphoinositides, and can direct the *in vitro* degradation of PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 , to PI, PI(4)P and PI(4,5)P_2 (Myers *et al* 1998).

Structurally PTEN bears sequence homology to other dual specificity phosphatases, and to the cytoskeletal protein, tensin (Liaw *et al* 1997). Homologous mutation in the *PTEN* gene, which is situated on human chromosome region 10q23, has been identified in many tumours including prostate cancer and gliomas. Mutation in a single copy of the gene results in Cowden disease and Bannayan zonana syndrome, which lead to a predisposition to certain tumours (Celebi et al1999). Recent evidence suggests that the ability to dephosphorylate PI(3,4,5)P_3 may contribute to PTEN's tumour suppressor activity (Stambolic *et al* 1998). Indeed, PTEN knockout mice exhibit elevated levels of PI(3,4,5)P_3 and enhanced PKB activity, and whilst biochemical analysis of tumours from

PTEN mutants could not detect any 3'-phosphatase activity, some protein phosphatase activity towards poly-Glu-phospho-Tyr was retained (Furnari *et al* 1998).

MYOTUBULARIN

A further regulator of the accumulation of PI3K products is Myotubularin which has been recently demonstrated to possess 3-phosphatase activity. Myotubularin was previously identified as a tyrosine phosphatase involved in muscle cell differentiation. Mutations in the MTM1 gene, which encodes this protein, have been demonstrated as the causative factor in X-linked myotubular myopathy (Wallgren *et al* 1995). Studies have implicated myotubularin in the regulation of PI(3)*P* accumulation, a crucial lipid which plays a role in intracellular membrane trafficking (Taylor *et al* 2000). Overexpression of a substrate trapping mutant of myotubularin led to the accumulation of elevated levels of PI(3)*P* in mammalian cells (Taylor *et al* 2000).

RUK

The novel cytosolic adaptor protein Ruk has recently been identified to negatively regulate pathways mediated via PI3K (Gout *et al* 2000). Ruk was first identified as an SH3 domain binding protein (Akopian *et al* 1996), and has been more recently shown to bind the p85 regulatory subunit of PI3K. This interaction is mediated via a proline rich region within the Ruk protein which associates with the SH3 domain of p85 (Gout *et al* 2000). Studies in which Ruk was overexpressed demonstrated that p85 and RUK co-association upregulated apoptosis, and that this could be reversed via the overexpression of catalytically active PI3K (p110α) or constitutively active PKB (Gout *et al* 2000).

1.2 PI3K EFFECTORS

The 3' -phosphatidylinositol lipid products of class 1A PI3Ks specifically target distinct classes of proteins which possess lipid binding motifs. One such motif is the FYVE domain which binds PI(3)*P*. A further class of PI lipid binding domains that shall be discussed here are pleckstrin homology domains.

1.2.1 CLASSIFICATION OF PH DOMAINS

PH domains are globular protein domains of about 100 amino acids that can bind phospholipids (Lemmon *et al* 1997). About 110 PH domain containing proteins have been described to date all of which, despite displaying divergent amino acid sequence, contain a conserved core fold consisting of a seven stranded β-barrel capped on one end by a C terminal α-helix (Rebecchi *et al* 1998). 10% of the PH domains that have been identified

are able to bind phosphoinositides with sufficient affinity to mediate the receptor driven membrane translocation of their host proteins, which elicits their activation (Kavran *et al* 1998, Lemmon *et al* 2000). PH domains that bind phosphoinositides with high affinity have been identified to have a conserved N terminal $KX_{7\ 13}$R/K X R ✣ motif, (where x is any amino acid and ✣ is a hydrophobic amino acid) the basic residues of which specifically interact with phosphorylated inositol head groups (Bottomley *et al* 1998). Of these a subset have been predicted to show preferential binding for the rare 3'-phosphoinositide products of PI3K (Isakoff *et al* 1998), which even after maximal receptor stimulation are still present at levels well below those of their abundant phospholipid precursors PI(4)*P*, and PI(4,5)P_2 (Auger *et al* 1990). To date, the PH domain containing Tec Kinase BTK (Fukuda *et al* 1997) and the ARF exchange factor Grp1(Klarlund *et al* 1997) have been shown to exclusively bind PI(3,4,5)P_3, whilst *in vitro* PKB has been shown to have affinity for PI(3,4,5)P_3 and PI(3,4)P_2 (James *et al* 1996). More recently

PI(3,4,5)P_3	**PI(3,4)P_2 AND PI(3,4,5)P_3**
Grp1	DAPP1
BTK	PKB
Cent-α	SBf1
DOS	A4054961
Gab1	
Gap1$_{IP4}$	
Gap1m	
PDK-1	

SIGNATURE MOTIF FOR 3'PHOSPHOINOSITIDE BINDING

[LVIMF]-X-**K**-X-[GASP]-X_m-(**K/R**)-X-**R**-X-[FL]-X-[LM]-X_n-[LIF]-X-**Y**

Table 4: predicted 3' phosphoinositide binding preferences of PH domain containing proteins (Ferguson *et al* 2000), showing the signature motif for 3'-phosphoinositide binding that has been recently defined,(x= any amino acid, m=1-5, n=6-10) (Lietzke *et al* 2000).

predictions for the preferential binding of PI(3,4,5)P_3 alone or PI(3,4,5)P_3 and PI(3,4)P_2, have been made for other PH domain containing proteins (Lietzke 2000, Ferguson *et al*

2000) (see table 4). A more detailed description of PI3K effector pathways that are mediated by PH domain containing proteins which possess either enzymatic function, or act as adaptor molecules, is described below:

1.2.2 THE PI3K/ TEC FAMILY KINASE PATHWAY

Important structural components of the Tec family kinases are summarised in diagram 2. The Tec family kinases target PLCγ (Sommer *et al* 1999, Schaeffer 1999) and are thus essential components of the intracellular signalling pathways leading to calcium mobilisation (Scharenberg *et al* 1998). Receptor stimulated calcium mobilisation is a product of PLCγ activation which in turn catalyses phosphatidylinositol (4,5)-bisphosphate (PIP_2) breakdown to yield di-acyl glycerol (DAG) and inositol (1,4,5)-tris phosphate (IP_3). IP_3 stimulates release from intra-cellular calcium stores in the ER which rapidly opens IP_3 sensitive store operated calcium (SOC) channels on the cell surface allowing calcium influx from the extra-cellular space (Putney *et al* 1993). Recent work has shown that Tec kinases can physically associate with components of the transmembrane adaptor protein LAT which also recruits PLCγ (Bunnell *et al* 2000) and SLP-76 (Su *et al* 1998), which complexes with LAT via Gads (Fluckiger *et al* 1998, Liu *et al* 1999).

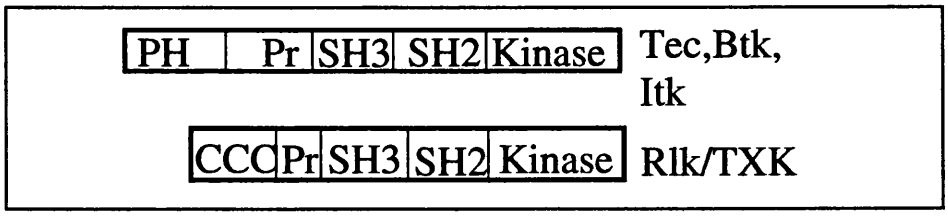


DIAGRAM 2: Structurally, the Tec family kinases comprise a single SH2 and SH3 domain and an upstream conserved region which has been denoted as the Tec homology region (TH). The TH region is characterised by a GTPase activating protein (GAP)-BTK homology (BH) region, and a proline rich sequence implicated in SH3 domain binding and kinase activity (Andreotti *et al* 1997). ITK, Tec, and BTK each exhibit a PH domain (Lemmon *et al* 1996), which is substituted in Rlk/Txk by an alternative cysteine rich region that acts as a site for palmitoylation and determines Rlk/TxK subcellular location at membrane vesicles (Debnath *et al* 1999). Activation of Tec kinases occurs upon association of 3'-phosphoinositides with the PH domain (August *et al* 1997), which allows the subsequent phosphorylation of tyrosine Y^{551} (Heyeck *et al* 1997).

1.2.3 THE PI3K/PKB PATHWAY

Protein kinase B (PKB) was first identified in 1991 as a PH domain containing serine threonine kinase which bore considerable homology within its kinase domain to PKCε and

PKA (Coffer *et al* 1991, Jones *et al* 1991, Bellacossa *et al* 1991). PKB's PH domain can preferentially bind PI(3,4)P_2 and PI(3,4,5)P_3 (James *et al* 1996, Stephens *et al* 1998), and has been shown to mediate PKB's rapid and transient membrane relocalisation upon the activation of PI3K (Andjelkovic *et al* 1997). In accordance with this observation, membrane localised PI3K constitutively upregulates PKB (Klippel *et al* 1997) and PKB activation is sensitive to the PI3K inhibitor wortmannin (Burgering *et al* 1995, Reif *et al* 1997). The binding of 3'-phosphoinositides to the PH domain of PKB induces a

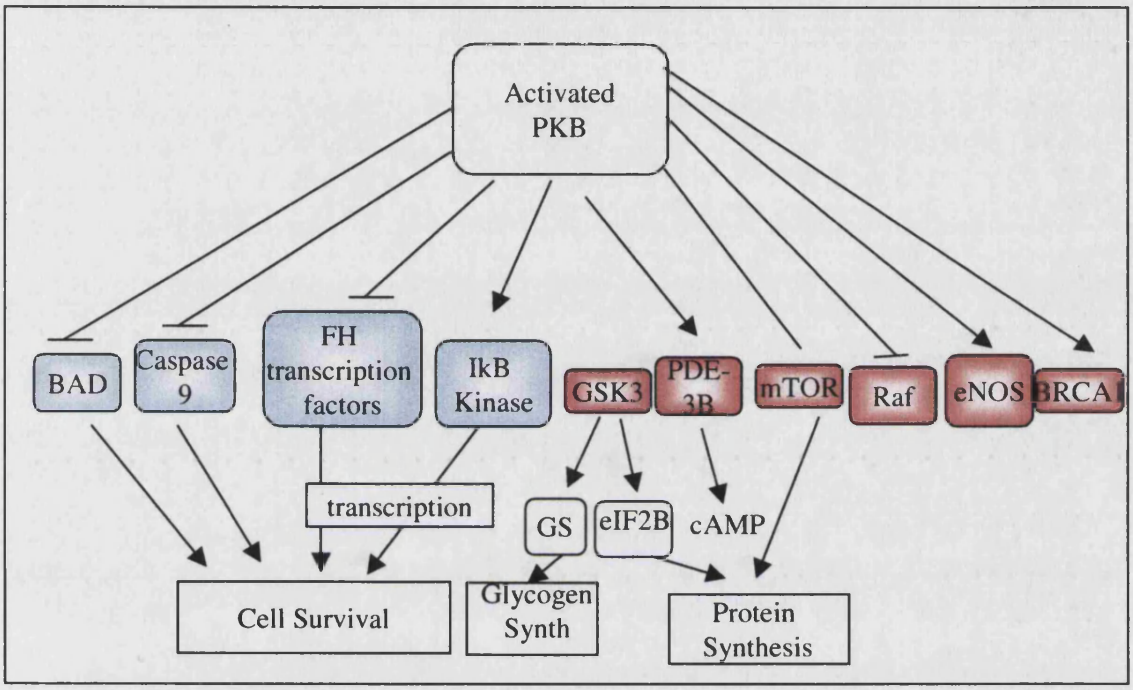


DIAGRAM 3: PKB Targets

conformational change, allowing it to become fully activated by phosphorylation of two conserved residues, threonine^{308} and serine^{473} (Alessi *et al* 1997). Phosphorylation of Thr^{308} is elicited by the activity of a further PH containing protein kinase, 3'phosphoinositide dependent kinase (PDK1), and is entirely dependent on the presence of PI(3,4)P_2 or PI(3,4,5)P_3 *in vitro* (Alessi *et al* 1997). The kinase which elicits Ser^{473} phosphorylation of PKB is as yet unidentified, and was putatively termed PDK2 (Alessi *et al* 1997). Integrin linked kinase (ILK) has been implicated in the phosphorylation of Ser^{473}, (Delcommenne *et al* 1998), but this is possibly via an indirect mechanism (Lynch *et al* 1999). Recent observations indicate that PDK2 may in fact be PDK1: PDK1 has been demonstrated to associate with what has been termed the PDK1 interacting fragment (PIF) of PKC related kinase 2 (PRK2), and this interaction allows PDK1 to phosphorylate Ser^{473} and Thr^{308} (Balendran 1999). Interestingly, this PRK2-PIF modified form of PDK1

demonstrates a 3 fold enhanced activity in the presence of PI(3,4,5)P_3 or PI(3,4)P_2 (Balendran 1999).

PKB plays an important role in the maintenance of cell survival in fibroblasts and epithelial cells (Coffer *et al* 1998, Bellacossa *et al* 1998). PKB targets proteins containing the amino acid sequence RXRXXS/T ✣ where X s any amino acid and ✣ is a bulky hydrophobic amino acid) (Alessi *et al* 1996). PKB's role in enhancing cell survival may be mediated via multiple pro-apoptotic proteins targets. PKB phosphorylates BAD, a member of the BCL2 family causing its dissociation from Bcl-2 and Bcl-X_l (Datta *et al* 1997, Yang *et al* 1999). PKB also targets forkhead transcription factors, resulting in their translocation from the nucleus (Brunet *et al* 1999, Kops *et al* 1999). PKB mediates the transcriptional regulation of anti-apoptotic factors, through the PKB elicited phosphorylation of I- κB kinase-α (IKKα) (Ozes *et al* 1999). IkB kinases phopshorylate IκB which causes its degradation and dissociation from NFκB, allowing NFκB translocation into the nucleus where it upregulates transcription. Further targets of PKB are summarised in the diagram above (Diagram 3).

1.2.4 PH CONTAINING ADAPTOR PROTEINS

The identification of novel cytosolic proteins which lack enzymatic function and have p85 binding capacity has led to the proposal that PI3K may be regulated via recruitment to different protein complexes by these 'adaptor' proteins. A few of these proteins relevant to this study are described below:

p62 DOK-1

The adaptor protein P62-Dok-1 has been described to associate with p85 and IRS-1 in the context of insulin mediated signalling pathways (Noguchi *et al* 1999). P62-Dok-1 was first identified as a cytosolic adaptor protein which sequestered the GTPase activating proteins (GAP's), Ras-Gap away from Ras. GAPs activate the intrinsic GTPase activity of Ras, and thus enhance GTP hydrolysis, reverting Ras to its inactive GDP bound conformation (McCormick 1993). Thus sequestration of Ras GAP by adaptors such as p62-Dok-1, inhibit the activation of Ras GTPase activity, and allow its accumulation in an active state (Yamanashi *et al* 1997).

GAB FAMILY ADAPTOR PROTEINS

Gab1 and Gab2 are cytosolic docking protein which have been demonstrated to associate with Grb2, Shc and the regulatory PI3K subunit p85 (Craddock and Welham 1997, Gadina 1999, Nishida *et al* 1999). Gab1 is the mammalian homologue of the drosophila protein Dos, that is coupled to sevenless and torso signalling via corkscrew (Herbst *et al* 1996).

Gab1, Gab2 and Dos each have PH domains, but elsewhere Gab2 displays only 37% homology to Gab1. Of functional note Gab1 and Gab2 share conserved tyrosine based motifs, which can bind the SH2 domains of PI3K, Crk, and SHP2. Gab1 and Gab2 also possess a conserved proline rich region which can associate with the grb2 SH3 binding motif. Additionally both Gab1 and Gab2 display a c-met binding domain (MBD (see diagram 4).

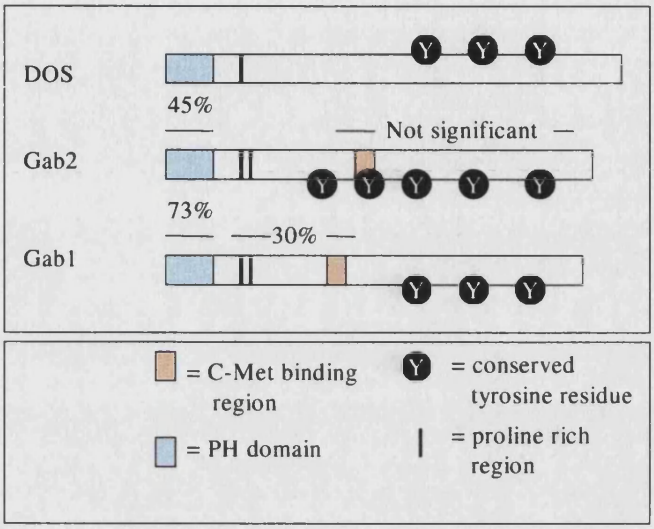


Diagram 4: Schematic representation of structural components of Gab1, Gab2 and Dos.
Dos, Gab1 and Gab2 each display a PH domain and several proline rich regions. Gab1 and Gab2 share a c-Met binding domain, and share 30 % amino acid identity outside of the PH domain. Amino acid identity is marked between each protein, conserved phosphotyrosine residues indicated are each within recognised SH2 binding motifs.

The PH domains of Gab1 and DOS have been predicted to preferentially bind PI(3,4,5)P_3 (Ferguson *et al* 2000). The structural similarity shared by the PH domains of this protein family might predict that Gab2 may also display a preference for binding PI(3,4,5)P_3 which would have implications in the kinetics of membrane recruitment of this protein. It has been demonstrated that overexpression of Gab1 and Gab2 can enhance Erk2 activation (Nishida *et al* 1999). Gab2 has been proposed to act as a substrate for SHP2 PTPase activity and this is supported by its reported hyper phosphorylation in the presence of enzymatically inactive SHP2 (Gu *et al* 1997) and reduced phosphorylation in SHP2 immuno-precipitates from stimulated cells (Craddock and Welham 1997). Furthermore, Gab2 is proposed to link SHP2 to the MAP kinase pathway (see diagram 5) and studies have demonstrated that disrupting Gab2 binding sites for SHP2 SH2 domains inhibits immediate early gene expresssion (Gu *et al* 1998), whilst enhancing MAP kinase activation (Zhao *et al* 1999, Gu et al 1998). In a more recent study, Gab2's coassociation with p85 has been shown to be essential for IL-3 dependent activation of the PI3K /AKT pathway (Gu *et al* 2000). In the same study Shc was implicated in mediating tyrosine phosphorylation of Gab2 and initiating a shc/grb2/Gab2/PI3K/AKT pathway in response to

IL-2 receptor signalling (Gu *et al* 2000). A role for Gab2 in mediating positive signals in T cells has also been described by studies which have shown that IL-2 and IL-15 receptor stimulation can couple to MAPK activation via Jak3 elicited tyrosine phosphorylation of Gab2 (Gadina *et al* 2000).

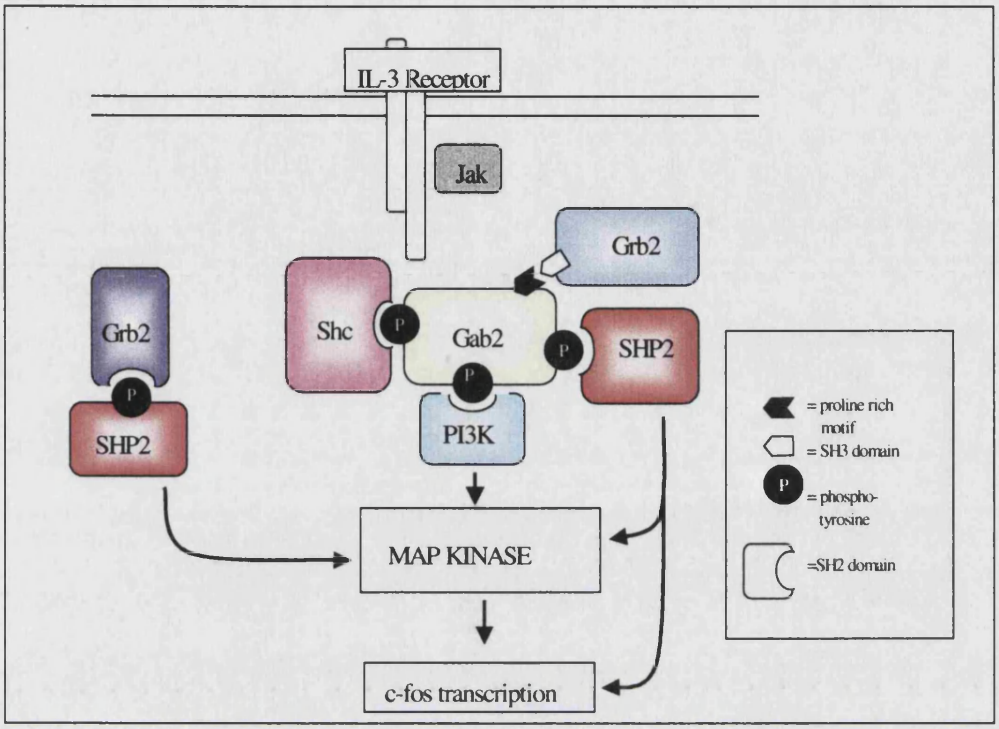


Diagram 5: Involvement of Gab2 in MAPK activation downstream of IL-3. Ligation of IL-3 receptor leads to activation of Jak s and tyrosine phosphorylation of SHP2 and Gab2. Gab2 may couple SHP2 and PI3K to MAPK activation but may enhance early gene expression via a parallel pathway through SHP2 as abolition of SHP2/ Gab2 interaction site inhibits Elk-1.

1.3 T AND B LYMPHOCYTES

1.3.1 THE IMMUNE SYSTEM

Cells of the T and B Lymphocyte lineages convey the specificity of the immune response. This specficity is attributable to the expression of a diverse repertoire of high affinity receptors for antigen on the surface of all but the most immature of the T and B lymphocyte population. T and B lymphocyte lineages arise via haemopoiesis, and develop from common lymphoid progenitor stem cells in the bone marrow, which differentiate into immature T and B Lymphocytes (Hsu *et al* 1983)

1.3.2 T LYMPHOCYTES

Immature T lymphocytes migrate to the thymus where they are 'educated' to discriminate between self and non-self via a process termed positive and negative selection. Furthermore, the expression of the CD4 or CD8 T lymphocyte surface antigens occurs in the thymus, culminating in the release of two distinct T cell subsets, $CD4^+$ and $CD8^+$, from

the thymus into the peripherary (Fowlkes *et al* 1989), resulting in a population of mature T cells that are non-responsive to antigens derived from self-expressed tissues,. Autoimmune diseases arise in individuals where incomplete negative selection results in circulating T cells that have the capacity to respond to self antigens (Blackman *et al* 1990).

1.3.3 B LYMPHOCYTES

Divergence of pre-B cells from the lymphoid progenitor cell population, and commitment to the B cell lineage, correlates with upregulation of the expression of the B cell surface receptor CD19. Expression of surface immunoglobulin (mIgM), otherwise known as the B cell antigen receptor (BCR), marks the emergence of the immature B cell from the bone marrow. At this stage the expression of the B220 surface antigen at low levels can be measured, and later in this stage CD21, the CD3dg complement receptor, is detected on human cells (Sato *et al* 1997). The mature B cell population is non – reactive to self expressed antigens. This is achieved in part by the clonal deletion of immature B cells which express B cell receptors which have high affinity for host proteins (Goodknow *et al* 1992).

1.4 T LYMPHOCYTE ACTIVATION

1.4.1 THE 2 SIGNAL MODEL

Naïve T cells require two signals to become fully activated and elicit a productive immune response: The first (signal 1) is delivered by the antigen specific T cell receptor (TCR)/ CD3 complex, upon encounter with foreign antigen bound by self major histocompatibility complexes (self MHC) on antigen presenting cells (APCs) (Bretscher *et al* 1992). Signal 2 is generated via costimulatory molecules that bind counter receptors expressed on the surface of antigen presenting cells (Schwartz *et al* 1990). An important and well characterised costimulatory molecule is CD28, a homodimeric glycoprotein, whose activation is triggered upon ligation with the APC expressed family of molecules, B7 (namely B7.1 (CD80) and B7.2 (CD86) (Guinan *et al* 1994).

The association of the TCR with antigen and CD28 with B7.1/B7.2 allows synergistic activation of pathways which control progression of the cell cycle from G0 to G1, up-regulation of the IL-2 receptor and secretion of IL-2 (Ward *et al* 1996). In addition, CD28 also promotes cell survival by up-regulation of Bcl X_L, and regulates expression of a number of other cytokines, chemokines, and chemokine receptors (Boise *et al* 1995, Labuda *et al* 1998). In the absence of a costimulatory signal, TCR stimulation is insufficient for proliferation of the cell to occur. Instead the T cell becomes anergic, characterised by non-responsiveness to further antigenic encounter, and may undergo

programmed cell death. If costimulation is provided in the absence of TCR engagement, drastically reduced cytokine production can occur. Optimal levels of cytokine production can be restored in this situation when phorbol esters are used in place of a TCR/CD3 signal (Bretscher *et al* 1992). Furthermore, CD28 engagement enhances the expression of other T cell expressed costimulatory molecules, such as CD40L which has been shown to be necessary to provide the full complement of signals that are able to activate naïve $CD8^+$ T cells and effector functions (Bennett *et al* 1998).

Thus, it is proposed that CD28 can provide the primary costimulatory signal. Evidence which supports this hypothesis can be drawn from the following studies:

i) Blocking B7/CD28 interaction by the use of F(Ab) fragments of CD28 antibodies causes cells to enter a state of hyporesponsiveness, strongly suggesting that it is through this interaction that T cell reactivity is initiated and maintained (Damle *et al* 1988).

ii) T cells from mice deficient in CD28 show decreased cytokine production and proliferation in response to mitogenic stimuli, again highlighting the importance of this molecule in T cell costimulatory pathways (Shanhinian *et al* 1993).

In the following section I shall expand on the key components which characterise the downstream biochemical events mediated firstly by the TCR and then the costimulatory receptor CD28

1.5 TCR/CD3 AND THE ANTIGEN DEPENDENT SIGNAL

1.5.1 TCR/CD3 STRUCTURE

Signal 1, the antigen dependent signal for T cell activation, is mediated via the TCR/CD3 complex. The TCR comprises the hyper-variable dimeric α and β chains, which provide the basis for antigen specificity, which are non covalently associated to the TCR ξ chain dimers. This multimeric complex is further coupled to the invariant CD3 γ, δ, and ε, chains (Izquierdo *et al* 1992, Bolen *et al* 1995). The initiation of antigen receptor signalling is also accompanied by the clustering of other T cell surface molecules, which include CD4, CD8, CD2 and LFA1. These proteins form non covalent associations with the CD3 ε and TCR ξ chains in the cytoplasm (Bolen *et al* 1995).

The intracytoplasmic tail of the TCR ζ chains contain three copies of an immunoglobulin tyrosine activation motifs (ITAM) sequence (D/E)XXYXX(I/L)6-8YXX(I/L), which is singly represented in each of the CD3 ε γ and δ chains (Reth *et al* 1989). ITAMs are specific targets for antigen receptor activated protein tyrosine kinases (PTKs), and provide

phosphotyrosine docking sites for Src homology (SH2) domain containing proteins (Chan *et al* 1994).

1.5.2 TCR ACTIVATED PTKS

The most proximal event to occur following ligation of the TCR by antigen is the activation of the src family PTKs, $p59^{fyn}$ and $p56^{lck}$. These PTKs elicit phosphorylation of the TCR/CD3 ITAMs, which in turn allows the recruitment, and activation, of Zap-70 (Osman *et al* 1995, Sunder-Plasman *et al* 1997, Weiss *et al* 1995) and Syk tyrosine kinases (Chu *et al* 1999).

$p56^{lck}$ has been described to associate via its SH3 domains with the proline rich region of the PI3K regulatory subunit p85 (Kapeller *et al* 1994, and Pleiman *et al* 1993) and, more recently, with SLP-76 (Sanzenbacher *et al* 1999). Studies in $p56^{Lck}$ deficient cell lines have demonstrated that TCR induced phosphoinositide hydrolysis, calcium mobilisation, and phosphorylation of PLCγ1 are $p56^{lck}$ dependent. Mutation of the SH3 domain of $p56^{lck}$ inhibited Ras/Map kinase activation, elevation of Erk or Mek phosphorylation or ERK induced CD69 surface expression (Sanzenbacher *et al* 1999). In adddition to its SH3 domain binding partners, $p56^{lck}$ can also bind to Zap70 via its SH2 domain (Neumeister *et al* 1998). An essential role for $p59^{fyn}$ in T Lymphocyte signalling has been described following studies in $p59^{fyn}$ knock out mice which show a similar phenotype to $p56^{lck}$ deficient mice and are blocked at the double negative ($CD4^-$, $CD8^-$) stage of thymocyte development (Zhang *et al* 1999). Previous studies have examined $p56^{fyn}$'s role in TCR signalling and have demonstrated a weak association between the TCR ζ chains and an N terminal region of $p56^{fyn}$ (Gauen *et al* 1992), which correlates with a 2-4 fold increase in $p56^{fyn}$ activity (Tsygankov *et al* 1992).

In response to TCR ligation, Zap-70 is phosphorylated in the interdomain B region, situated between the kinase domain and the C- terminal SH2 domain, on Y^{319}. (Di Bartolo *et al* 1999, and Williams *et al* 1999). Activation of Zap 70 is crucial for CD69 elevation, PLCγ phosphorylation (Williams *et al* 1999), NFAT transcription and IL-2 production (Di Bartolo *et al* 1999). Zap-70 mutant T cells are unable to mediate Ca^{2+} mobilisation and MAPK activation (Shan *et al* 1999).

1.5.3 TCR MEDIATED EFFECTOR PATHWAYS.

REGULATION OF RAS

TCR ligation induces the accumulation of the monomeric guanine nucleotide binding proto-oncogene product, Ras (Downward *et al* 1990), in its active GTP bound conformation. Ras activation is critical during thymocyte and B cell development (Gartner *et al* 1999), whilst in mature lymphocytes Ras is essential for proliferation and cytokine gene transcription (Turner *et al* 1998). Ras activity is positively regulated in T cells by the guanine nucleotide exchange factor SOS (GEF's), which elicit accumulation of Ras in its active GTP bound form. SOS is activated upon membrane recruitment by formation of Shc/Grb2 complexes (Holsinger *et al* 1995). The SH2 domains of Grb2 associate with tyrosine phosphorylated Shc, whilst Grb2s' SH3 domain can bind a proline rich region on SOS (McCormick *et al* 1993).

The recent identification of the transmembrane adaptor protein LAT which recruits TCR regulated signalling complexes, has further characterised the events which couple TCR ligation to Ras activation. The TCR stimulated accumulation of GTP-bound Ras leads to the activation of Ras effectors, namely PI3K, (Rodriguez-viciana *et al* 1997), Raf , Rac and Ral GDS (Marshall *et al* 1996 and 1999), and activates downstream targets such as the transcription factors of the NFAT (Nuclear factor of activated T cells) family, (Angel *et al* 1991, Genot *et al* 1996), and AP-1, Elk-1 and serum response factor-1 (Turner *et al* 1997), via Mitogen activated protein kinase cascades (MAPK) (Blenis *et al* 1993). Interestingly, data also exists which places Ras downstream of PI3K (Hu *et al* 1995). TCR initiated signals are insufficient to activate the Rac effector, c-Jun N terminal kinase (JNK) that enhances transcriptional activity of c-jun and thus assembles AP-1 transcription factor complexes (Jain *et al* 1995). Signal 2 CD28 costimulatory signals are required for transcriptional activation via this pathway (Su *et al* 1994) and are discussed later (see diagram 8).

TCR ACTIVATION OF PI3K AND ITS EFFECTORS

In T cells, TCR stimulation by antigen ligation activates class 1A PI3Ks. The candidate adaptors for recruiting PI3K to the membrane in T cells include the TCR associated adaptor protein TRIM (Bruyns *et al* 1998) and LAT (Zhang *et al* 1998). Membrane recruitment of PI3K may elicit its activation via protein kinase activity, and previous studies have shown that in T cells serine/threonine kinases mediate phosphorylation of PI3K downstream of the TCR (Reif *et al* 1993, Ward *et al* 1992). Alternatively, approximation of PI3K with Ras could be the predominant route for PI3K activation in T

cells (Zhang *et al* 1998). However, PI3K dependent pathways leading to PKB activation can be activated independently of Ras in T cells (Genot *et al* 1998).

Targets of TCR activated PI3K include the PH domain containing proteins ITK (Scharenberg *et al* 1998, Bolland *et al* 1998) and Akt/PKB (Reif *et al* 1997). ITK has been firmly implicated in PLCγ1 phosphorylation and calcium signalling downstream of antigen receptor signals (Schaeffer *et al* 1999, Sommers *et al* 1999). However one report which examined ITK activation following TCR stimulation suggests that this is not a PI3K dependent event (Heyeck *et al* 1997). PKB may promote cell cycle progression in T cells, through the stimulation of E2F and its subsequent promotion of Cyclin D3 expression which drives G1 to S transition (Brennan *et al* 1997).

1.6 CD28 AND B7 FAMILIES

1.6.1 CD28

CD28 is a 44 kDa homodimeric glycoprotein which is constitutively expressed on 95% of CD4+ T cells and 50% of the CD8 compartment. It contains a single disulphide linked IgV like domain making it a member of the Ig superfamily. The mature CD28 polypeptide contains 202 amino acids resulting in a protein of 23 kDa which is then glycosylated to 44kDa. A single transmembrane pass links the extracellular region to a 41 amino acid cytoplasmic tail which exhibits no catalytic activity (June *et al* 1994). The intracytoplasmic tail of CD28 exhibits four highly conserved tyrosine residues (see diagram 6), one of which, at ^{173}Y, is situated in a conserved motif YXMN (where X is any amino acid) and which in its phosphorylated state has specificity for the SH2 domains of the phosphoinositide 3-OH kinase (PI3K) regulatory sub-unit, p85 (Ward *et al* 1993, Pages *et al* 1994). Further studies have identified two proline rich motifs, which have been shown to be essential for costimulation (Pages *et al* 1994, Truitt *et al* 1996).

CD28 co-ligation with the TCR results in IL-2 receptor expression and IL-2 secretion. Costimulatory signals through CD28 initiate the production of cytokines IL-4, IL-8, IL-13 and GMCSF (Weschler *et al* 1994). CD28 also leads to the activation of transcriptional transactivators NFκB and AP-1, and messenger RNA stabilisation of cytokine transcripts. Chemokine expression is also thought to be upregulated by CD28 via NFκB (Shapiro *et al* 1997, Moriuchi *et al* 1997, Edmead *et al* 1996).

1.6.2 ICOS

ICOS is another member of the CD28/CTLA-4 family which plays an important role in production of IL-2, IL-4, IL-5, and IFN γ from recently activated T cells (Coyle *et al*

2000). ICOS displays structural similarity to CD28 and CTLA4, but lacks the HYQPY motif SH3 domain binding motif, which indicates that it recruits alternative proteins. Recent reports have shown that, rather than binding B7-1 or B7-2, ICOS binds a new B7 related molecule of previously unknown function called

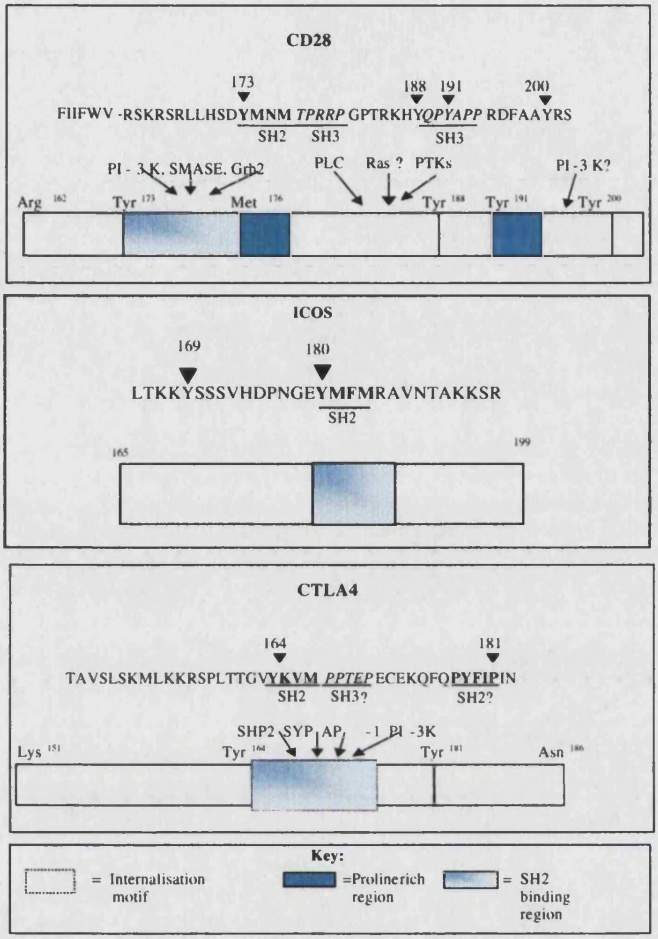


Diagram 6: Schematic showing CD28, ICOS and CTLA4 amino acid sequences and structural motifs.

LICOS (ligand of ICOS) or B7.h (Brodie *et al* 2000). Crosslinking ICOS has been demonstrated to enhance T cell proliferation and cytokine production particularly of TH2 T cell responses leading to IL-10 production(Hutloff *et al* 1999).

1.6.3 CTLA4

Cytotoxic T Lymphocyte antigen 4 (CTLA4) shares an overall 31% amino acid homology with CD28, with 36% homology displayed by their respective intracytoplamic tails (Freeman *et al* 1993). Although initially thought to deliver supplementary costimulatory signals, CTLA4 provides a natural 'braking' mechanism which inhibits T cell activation (Krummel *et al* 1995, Walanus *et al* 1996). CTLA4 is expressed as a homodimer of a 20kDa protein at low levels on the surface of $CD4^+$ T cells, whilst slightly higher levels are detected on $CD8^+$ T cells (Walunas *et al* 1999). CD28 ligation of CD3 activated cells

transcriptionally upregulates CTLA4, with surface protein expression appearing at 48 hrs post activation (Blair *et al* 1998). CTLA4 shares specificity with CD28 for the B7 family of ligands, with which CTLA4 can associate at a 10 fold higher affinity, and a 100 fold greater avidity than CD28 (Van der Merwe *et al* 1997).

CTLA4 contains a core internalisation motif of 9 amino acids which is a tyrosine based consensus motif (TGVYVKM) to which a number of proteins are recruited e.g. PI3K (Schneider *et al* 1995) SHP1, SHP2 (Myers *et al* 1996, Marengere *et al* 1996). Internalisation is mediated by AP-1, 2, and 3 adaptor proteins, which initiate endocytosis of CTLA4 in clathrin coated pits (Ohno *et al* 1998, Shiratori *et al* 1997, Pearce *et al* 1990) Internalisation is negatively regulated by tyrosine phosphorylation at Y^{165} by $p56^{lck}$ (Bradshaw et al 1997). Consequently, the majority of CTLA4 protein is intra-cellularly expressed, co-localising with the peri nuclear golgi apparatus, and upon activation trafficking to the plasma membrane at the site of TCR-APC ligation (Linsley *et al* 1996, and Leung *et al* 1995).

1.6.4 B7 FAMILY LIGANDS

Members of the B7 family of proteins were identified as physiological ligands for CD28 and CTLA4 (Linsley *et al* 1991a), and were demonstrated to bind CTLA4 with 20–100 fold higher affinity than the extracellular domain of CD28 (Linsley *et al* 1991b). B7.1 the first identified family member which was shown to provide co-stimulatory signals for antigen activated T cells (Linsley *et al* 1991a, Gimmi *et al* 1991) and B7.2 and B7.h have since been identified (Boussiotis *et al* 1993, Freeman *et al* 1993). B7.1 and B7.2 are Ig family members and share just 25% sequence homology (Linsley *et al* 1991,Guinan *et al* 1994). B7.1 is a 60 kDa glycoprotein (30 kDa polypeptide) which consists of the two extracellular disulphide linked Ig - like domains, a transmembrane region and a short cytoplasmic domain of 19 amino acids (June *et al* 1994). In contrast, B7.2 is a 70 kDa molecule, (34 kDa polypeptide glycosylated) with an extended cytoplasmic domain containing phosphorylation sites for protein kinase C (PKC), which may indicate a signalling role in APCs (Freeman *et al* 1993).

B7.2 expression has been detected on resting monocytes whilst expression of both B7 molecules is found on activated cells of the monocyte, B and T lymphocyte and natural killer cell lineages (Guinan *et al* 1994, Azuma *et al* 1993, Freeman *et al* 1993). Differing temporal regulation of B7.2 and B7.1 exists, with up-regulation of B7.2 surface

expression at 72 hours (Azuma *et al* 1993), whilst B7.1 is detected on the cell surface 3 days later (Freeman *et al* 1993). B7.h is a more recently identified member of the B7 family which is constitutively expressed and may mediate costimulation of ICOS leading to IL-10 secretion (Brodie *et al* 2000).

Multiple reports indicate that their may be different functional consequences of engaging CD28 by B7.1 as compared to B7.2. Whilst a role for B7.1 in mediating Th2 T cell responses is described, B7.2 may mediate Th1 T cell responses (Lenschow *et al* 1996, Matulonis *et al* 1996). B7.1 and B7.2, co-stimulate equivalent CD3/CD28 dependent NFAT mediated transcription, and each can induce phosphorylation of a similar array of proteins, including Vav and Cbl (Slavik *et al* 1999). However, B7.1 mediates more potent phosphorylation of CD28 and PLCγ1 and induces greater co-association between CD28/PI3K, than that observed following liugation by B7.2 (Slavik *et al* 1999).

1.7 THE COSTIMULATORY SIGNAL

The identity of the biochemical components of the costimulatory signal mediated by CD28 has been the focus of much research. Evidence exists which suggests that the pathway downstream of CD28, is distinct to TCR/CD3 triggered pathways. This notion is evidenced by the fact that the CD28 triggered signalling pathway is resistant to the immunosuppressant Cyclosporin A (CspA), but sensitive to rapamycin, whilst the TCR/CD3 pathway is blocked by CspA (June *et al* 1987). Further research has demonstrated that TCR and CD28 signals may activate unique biochemical targets e.g. the CD28 mediated phosphorylation of P62 DOK cannot be detected following TCR ligation (Klasen *et al* 1998). However the identification of common downstream targets of the CD28 and TCR signalling cascades may suggest that these signals are similar and that they act in an additive fashion. For example signal 1 and signal 2 both mediate the phosphorylation of Vav (Tusto *et al* 1996, Klasen *et al* 1998).

1.7.1 THE CD28 SIGNAL- IMMEDIATE UPSTREAM EVENTS

CD28 ligation results in the phosphorylation of four tyrosine residues within its cytoplasmic tail, mutation of which significantly impairs costimulation (Teng *et al* 1996). The identity of the PTK which mediate CD28 tyrosine phosphorylation is controversial. A recent study has shown that a $p56^{Lck}$ SH3 domain mediated association with the proline residues in the CD28 tail is essential for activation of $p56^{Lck}$ and costimulation, and that costimulation cannot occur in $p56^{Lck}$ deficient cells (Holdorf *et al* 1999). However previous studies which were carried out in $p56^{Lck}$ deficient cells showed that activation of PI3K

along with elevation of intracellular calcium levels ($[Ca^{2+}]$ i) could still be achieved and it was suggested that proximal events in the CD28 pathway are independent of src kinases and are therefore distinct from those mediated by the TCR (Lu *et al* 1994).

Other studies supported a role for src family PTKs in CD28 phosporylation as treatment of cells with herbimycin A, a src family tyrosine kinase inhibitor, caused the inhibition of of IL-2 production, PI3K association and the tyrosine phosphorylation of cellular proteins after TCR and CD28 stimulation (June *et al* 1994).

1.7.2 CD28 MEDIATED ACTIVATION OF PI3K AND ITS EFFECTORS

The first strong evidence which implicated a role for PI3K in T cell costimulatory pathways was the rapid and massive accumulation of D3 phospoinositides, in particular of PI(3,4,5,) P_3, upon CD28 stimulation (Ward *et al* 1996). Furthermore, pre-treatment of primary human T lymphoblasts with nanomolar concentrations of wortmannin, a fungal metabolite which irreversibly blocks PI3K activity, can inhibit CD28 dependent costimulation, resulting in decreased proliferation and IL2 production (Ward *et al* 1995). Further evidence from molecular studies supports the role of PI3K in mediating the costimulatory signal: disruption of the PI3K binding site within the CD28 cytoplasmic tail led to abrogated IL-2 production following TCR/CD28 costimulation of a T cell hybridoma cell line (Pages *et al* 1994).

However, several studies have disputed the importance of PI3K in CD28 mediated signalling pathways. One study suggested that PI3Ks major role was to mediate receptor endocytosis, as mutations that disrupt PI3K binding and co-stimulation prevented the internalisation of CD28 (Cefai *et al* 1998). Another group demonstrated that mutation of the PI3K phosphotyrosine binding site Y^{173} failed to block costimulation, whilst mutation of an alternative residue Y^{188}, abrogated IL-2 production (Sadra *et al* 1999). Furthermore, p85α deficient mice which demonstrate multiple B cell defects, exhibit no defects in T cell costimulatory repsonses (Suzuki *et al* 1999). Studies which have employed the acute lymphoblastic T cell line Jurkat, also demonstrated that wortmannin treatment failed to block IL-2 production (Crooks *et al* 1995) upon CD28 co-ligation with the TCR. The use of the Jurkat T cell line for functional studies is however questionable, as recent work has shown that this cell line lacks important regulatory lipid phosphatases (Edmunds *et al* 1999, Shan *et al* 2000).

CD28 /PI3K/TEC KINASE ACTIVATION

CD28 ligation stimulates the tyrosine phosphorylation of ITK and Tec and a physical interaction between TEC and ITK through an interaction between the proline rich motif of

the CD28 intracytoplasmic tail, and the SH3 domain of Tec or ITK has been detected (Lu *et al* 1998) Previous work has demonstrated that ITK has the capacity to elicit tyrosine phosphorylation of all four tyrosine residues in the CD28 cytoplasmic tail (King *et al* 1997). CD28 induced activation of $p56^{Lck}$ (Marengere *et al* 1997, and Yang *et al* 1999), and studies indicate that in response to CD28 ligation $p56^{Lck}$ phosphorylates ITK within the activation loop of ITK at Y^{511} (Heyeck *et al* 1997). Furthermore, $p56^{Lck}$ has been demonstrated to phosphorylate the PI3K interaction motif, Y 173 within the CD28 cytoplasmic tail (King *et al* 1997). CD28, but not CD3 mediated, ITK activation is PI3K dependent (Lu *et al* 1998). Taken together these reports suggest that the recruitment of ITK to the CD28 receptor and its phosphorylation and activation by $p56^{LCK}$ is dependent on the association of PI3K D-3 lipid products with the PH domain of ITK (Lu *et al* 1998). Indeed a physical association between ITK and p85 has been detected which appears to be mediated through the SH2 domain of ITK (Lu *et al* 1998). A proposed summary of the interplay between PI3K $p56^{lck}$ and ITK is depicted in diagram 7.

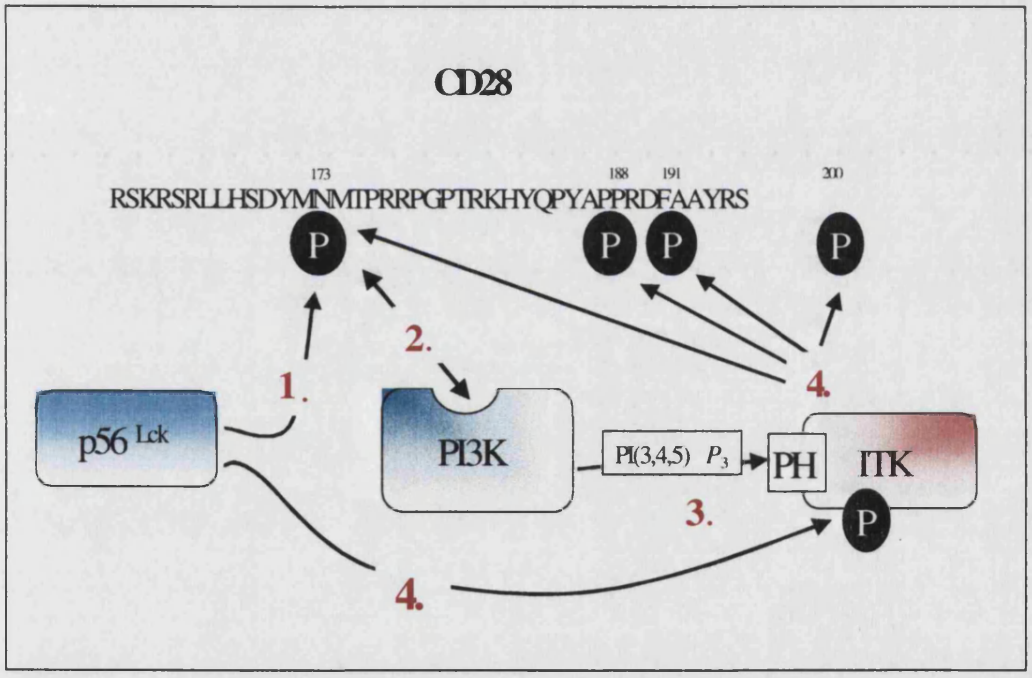


Diagram 7: Proposed sequence of events leading to phosphorylation of CD28 by ITK and $p56^{LCK}$
1. $p56^{Lck}$ phosphorylates Y 173 of the Cd28 cytoplasmic tail. 2. PI3K is recruited to Y173 and is activated. 3. PI(3,4,5)P_3 association with the PH domain of ITK recruits ITK to the membrane where it is activated by $p56^{Lck}$ and can phosphorylate Y173, Y 188, Y 191, and Y 200.

In strong support of differential regulation of ITK by CD3 and CD28 stimuli, a negative regulatory role for ITK in costimulatory signalling has been proposed, which contrasts with its role in augmenting phosphorylation of PLCγ following TCR ligation, (Liao *et al* al 1997). This suggestion is supported by the fact that ITK deficient T cells exhibit elevated *In vitro* proliferative responses following CD28 ligation (Liao *et al* 1995, Holdorf

et al 1998). However further data has shown that Tec kinases are involved in IL-2 and IL-4 transcriptional activaty in the CD28 pathway (Yang *et al* 2000)

CD28 / PI3K / PKB PATHWAY

Recent evidence has suggested that CD28 ligation leads to the PI3K dependent activation of PKB (Parry *et al* 1997). This, in addition to CD28 up-regulation of Bcl-X_l expression further illustrates CD28's role in enhancing resistance to apoptosis induced by irradiation, and CD3 or Fas Ab treatment. PKB has been implicated in the activation of E2F transcription factors (Brennan *et al* 1997), and CD28 has been implicated in cell cycle progression. CD28 co-ligation with CD3 upregulates G_1 cell cycle kinases (Nagasawa *et al* 1997), and the degradation of the cell cycle inhibitor p27Kip (Firpo *et al* 1994) which leads to transition from G_o to S phases of the cell cycle.

CD28 / PI3K / VAV GTP EXCHANGE ACTIVITY

The Rho family Guanine nucleotide exchange factor Vav is tyrosine phosphorylated upon CD28 ligation by $p56^{lck}$. Phosphorylated Vav couples to the C terminal SH2 domain of Grb 2 CD28 can mediate an association with Grb2 through the co-operative binding of the Grb2 SH2 and SH3 domains (Kim *et al* 1998) to the CD28 tail. Interestingly the Grb2 SH2 domain shares specificity with p85 for binding to the CD28YXXM motif, however Grb2 has been reported to interact with CD28 at extremely low stoichiometry (Ramos-Morales *et al* 1994). Accordingly, phosphorylated Vav is detected in association with Grb2 and CD28 following CD28 ligation (Kim *et al* 1998) and disruption of the Grb2 SH2 binding site (Gln^{193}) attenuates this complex (Kim *et al* 1998), which again correlates with a reduction in IL-2 production. CD28 can mediate phosphorylation of Vav at Y^{174} which enhances its GEF activity, and this is further promoted by the binding of PI(3,4,5)P_3 phosphoinositides to Vav's PH domain (Han *et al* 1998). Vav may therefore explain the PI3K dependency of CD28 mediated IL-2 production (Han *et al* 1998). Vav is also known to complex to the adaptor protein SLP 76 after TCR/CD3 and CD28 ligation, and thus it is thought that Vav can couple both of these receptors to Rac signalling in T cells (Han *et al* 1998).

In summary CD28 induced accumulation of GTP bound Rac can stimulate IL-2 transcriptional activation via JNK mediated assembly of AP-1 transcription factor complexes (See Diagram 8) (Su *et al* 1994). JNK is the anchor kinase of the Rac/ PAK/MEKK1/SEK/JNK signalling cascade.

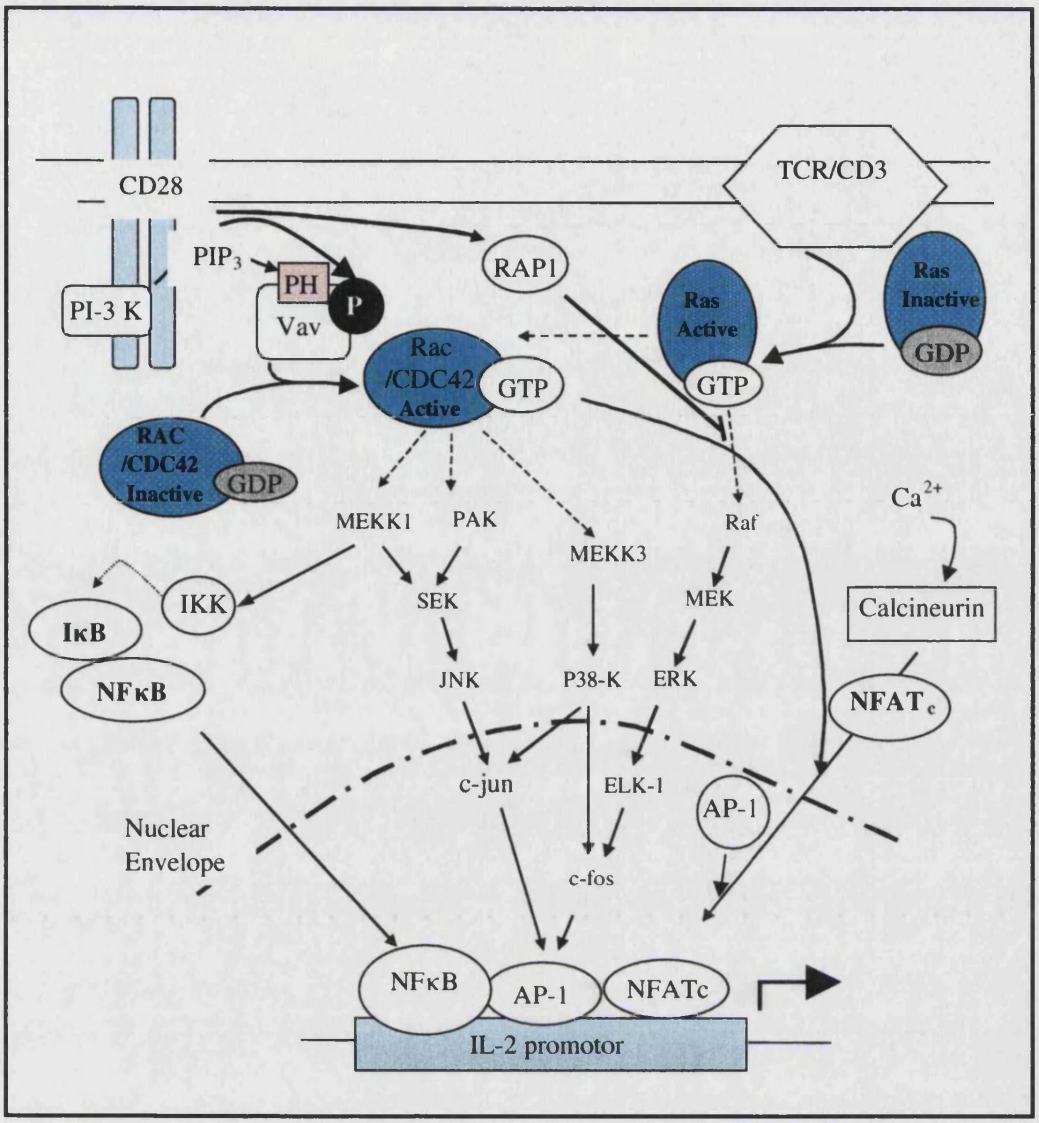


Diagram 8: IL-2 transcriptional activation.

TCR mediated signals couple to IL-2 transcription via the Raf1/MEK/ERK cascade MAPK cascade. ERK MAPK phosphorylates the ELK-1 transcription factor (a ternary complex factor, tcf) which activates serum response factor transcription (SRF), and together tcf and SRF stimulate c-fos transcription. CD28 mediated signals activate Rac GTP exchange factor Vav which leads to GTP Rac and GTP CDC42accumulation. In turn the recruitment of PAK and MEKK1 protein kinases leads to the activation of the PAK/MEKK1/SEK/JNK MAPK cascade. As CD3 mediated signals can also contribute to Rac activation, via Ras, the Rac/PAK/MEKK1/SEK/JNK cascade is a point of intersection between signal 1 and signal 2.

CD28 / PI3K / CYTOSKELETON AND SMACS

CD28 has been implicated in the cytoskeletal reorganisation which occurs upon T cell APC interaction. Upon initial T cell APC interaction weak associations between a ring of TCR and MHC, and CD28 and B7 complexes are stabilised by a high concentration of centrally located integrin molecules. Subsequent formation of strong affinity CD28 and B7, and MHC/TCR associations inverts the synapse structure such that integrin molecules encircle a centrally located group of tightly bound TCR/MHC complexes, which is accompanied by the initiation of intracellular signals (Shaw *et al* 1997, Dustin *et al* 1999). This complex has been termed SMAC (for supramolecular activation cluster). Glycolipid enriched motifs exist within this region to which the TCR and CD28 relocalise upon receptor ligation (James *et al* 1999). CD28 can induce major relocalisation of TCR complexes to the actin cytoskeleton in a PI3K and ATP dependent fashion and may potentiate the formation of SMACS (Viola *et al* 1999). Interestingly the only PKC isoform to translocate to SMACS is PKCθ which has been heavily implicated in CD28/CD3 costimulation, and interestingly may have a conserved docking site for the 3' phosphoinositide dependent kinase PDK-1 (Brennan *et al* 2000, Kane *et al* 2000). In addition to PKCθ, ZAP-70 and LAT (Lin *et al* 1999) re-localise to SMACS upon TCR/CD28 triggering (Wulfing *et al* 1998). LAT and Zap 70 have been implicated in CD28/CD3 costimulation: co-ligation of CD28 and the TCR results in increased tyrosine phosphorylation of LAT, whilst CD28 ligation leads to phosphorylation of LAT in a ZAP-70 and Syk independent manner, implicating LAT as a target, and most probably a mediator of costimulatory signals (Tsuchida *et al* 1999).

1.7.3 TRANSCRIPTIONAL REGULATION OF IL-2 BY CD28

THE CD28 RESPONSE ELEMENT

CD28 ligation via monoclonal abs or physiological ligand, in the presence of phorbol esters, can lead to cyclosporin A resistant IL-2 production (June *et al* 1994) (see diagram 8). The upstream regulatory sequences of the *IL-2* gene contain elements which confer CD28 responsiveness on IL-2 transcription (Civil *et al* 1992). These elements, located between –160 and –152 bp relative to the transcriptional start site (Fraser *et al* 1991), have been characterised and have been individually designated as the CD28 response element (CD28RE) and the NF-IL-2 and AP-1 sites (Civil *et al* 1992, Shapiro *et al* 1997).

The CD28 RE has been demonstrated to bind members of the NFκB family including c-Rel, NFκB1, and relA, (Ghosh *et al* 1993, Shapiro *et al* 1997). NFκB proteins enter the nucleus following CD28 stimulation. Furthermore, in response to costimulation, the binding of the transcriptional activator NFAT to this region can co-operate, with NFκB family DNA binding factors, in mediating transcriptional up-regulation of the IL-2 promoter, (Rooney *et al* 1995, Good *et al* 1996). Cytokine and chemokine genes encoding, IL-8 (Weschler *et al* 1994), RANTES (Nelson *et al* 1993) IL-3, granulocyte macrophage colony stimulating factor (GM-CSF) and gamma-interferon (IFNγ) (Fraser *et al* 1991) contain similar sequences to the CD28RE within their promoter regions, however these are less stringently CD28 dependent.

In addition to the factors that bind the CD28RE, CD28 mediated costimulation stimulates transcription of c-Jun, via the JNK cascade, which forms the AP-1 heterodimer with c-fos, and binds NF-IL2 sites within the IL-2 promotor (Rincon *et al* 1994). CD28 signals are alone insufficient to activate c-jun, and the integration of CD3 and CD28 signals, which can be replaced by phorbol ester and calcium ionophore treatment, is required (Su *et al* 1994). AP-1 binds the IL-2 promoter alone, at the NF-IL-2B site, in association with NFAT, at the NF-IL2E site, or with octamer proteins, at the NF-IL-2A site proteins (Shapiro *et al* 1997). Together, the CD28RE and the NF-IL-2B sites, which are separated by only 2bp, have been shown to synergistically regulate the CD28 mediated component of transcriptional activation of the IL-2 gene through the coordinated binding of c-Rel and AP-1 proteins, c-fos and c-jun (Shapiro *et al* 1997).

CD28 AND NFκB

CD28 also activates IκB kinase (IKK), which elicits the phosphorylation and degradation of the IκBα and IκBβ proteins that allows the release of NFκB proteins to the nucleus (Zandi *et al* 1997). It was demonstrated that CD28 regulates IKK via MEKK1 which colocalises with the IKK, IκB complex (Kempiak *et al* 1999). MEKK1, is a regulator of the Rac and p21 activated kinases/Mekk1/SEK/JNK p38 MAPK cascade, which converges on JNK (Kaga *et al* 1998). Thus, MEKK plays a dual role in regulating IL-2 transcription, in eliciting transcriptional activation of c-jun via JNK, and of c-Rel via IKK (Kempiak *et al* 1999). More recently studies have described further routes to IKK activation one of which is mediated via Cot Kinase which can activate IKKα, and NIK which in turn activates IKKβ (Lin *et al* 1999). Another pathway to IKKβ activation is via PKCθ (Lin et 2000), and a further pathway has been proposed which is PI3K/PKB dependent. It is

unclear as yet whether the latter pathway lies upstream of PKCθ and/or Cot kinase (Kane *et al* 2000, Brennan *et al* 2000).

1.8 THE T CELL INHIBITORY SIGNAL- CTLA4

CTLA4 has been clearly demonstrated *in vitro* to inhibit murine (Chambers *et al* 1997) and human (Blair *et al* 1998) T cell proliferation. CTLA4 engagement inhibits induction of IL-2 α receptor chain, CD69 expression and secretion of IL-2 (Chambers *et al* 1997). Characterisation of the inhibitory signal mediated by CTLA4 has been the focus of much research. The identification of SHP1 and SHP2 association with CTLA4 suggested a role for PTPases in CTLA4 function (Marengere *et al* 1996, Chambers and Allison 1996), which correlated with the observed hyperphosphorylation of TCR/CD3 signalling proteins: ZAP-70, CD3ζ chain, Shc, fyn and Lck, in T cells from CTLA4 deficient T cells. Further studies have demonstrated that CTLA4 ligation prevents the accumulation of IL-2 mRNA via the inhibition of NFAT translocation to the nucleus (Brunner *et al* 1999). CTLA4 has been shown to impair progression of the cell cycle through the inhibiton of the cyclin dependent kinases, cdk4 and cyclin D3, (Brunner *et al* 1996). Furthermore, TGFβ has been implicated in CTLA4 signalling through the observation that TGFβ and CTLA4 deficient mice exhibit similar proliferative disorder (Chen *et al* 1998). Recently, it has been demonstrated that CTLA4 can mediate inhibitory signalling independently of tyrosine phosphorylation (Cinek *et al* 2000, Baroja *et al* 2000). In a further study CTLA4 has been described to inhibit T cell signalling via two distinct mechanisms: In the presence of B7 costimulation, high levels of CTLA4 surface expression antagonise B7/CD28 interactions independently of the CTLA4 cytoplasmic domain; In the absence of B7 co-stimulation, low levels of CTLA4 expression mediate inhibitory signals which are dependent on the CTLA4 cytoplasmic tail (Baroja *et al* 2000).

1.8.1 CTLA4 DEFICIENT MICE

CTLA4 deficient mice exhibit a dramatic lymphoproliferative disease, which is lethal at 3-4 weeks of age (Tivol *et al* 1995). These mice exhibit a huge expansion of $CD4^+$ and, in particular the generally smaller $CD8^+$, T cell compartments (Waterhouse *et al* 1995) whilst thymocyte development is normal (Chambers *et al* 1997). The majority of peripheral T cells in these mice have an activated phenotype (Chambers *et al* 1996).

Further reports demonstrated that B7/CD28 interaction is necessary to induce the lymphoproliferative phenotype in these mice (Tivol *et al* 1997, Chambers *et al* 1997).

Antibody depletion of $CD4^+$ T cells in CTLA4-/- mice was shown to inhibit the lymphoproliferative effect observed and remaining $CD8^+$ cells displayed a normal phenotype, whilst $CD8^+$ depletion had no effect on the severity of the disease (Chambers *et al* 1997), which suggested that the lymphoproliferation was strongly CD4 dependent.

1.8.2 CTLA4-IMMUNOTHERAPEUTIC STRATEGIES

Immunotherapeutic strategies have been devised which exploit the regulatory role of CD28 in the immune system. The blockade of CTLA4 engagement *in vivo* exacerbates autoimmune disease, including autoimmune diabetes (Luhder *et al* 1998), graft versus host disease in $CD28^{-/-}$ mice (Saito *et al* 1998) and leads to allograft rejection (Lin *et al* 1998). Strategies, which disrupt CTLA4 inhibitory signals, and thus enhance tumour rejection in normal mice (Leach 1996), have been utilised, and the successful treatment of prostate tumours (Kwon *et al* 1997) and experimental mammary carcinoma (Hurwitz *et al* 1998) have been reported. CTLA4 blockade has also been shown to result in protective immunity to nematode infection (McCoy *et al* 1997), and Leishmania donovani (Murphy *et al* 1998), whilst CTLA4 has been shown to be necessary for tolerance induction to allogeneic skin grafts (Perez *et al* 1997).

1.9 B LYMPHOCYTE ACTIVATION

Activated B cells produce antibodies, which contribute to the 'humoral' immune response. Antibodies neutralise and eliminate the specific antigen which elicited their formation. The elimination of antigen relies on the combined efforts of the effector mechanisms which are elicited by the distinct isotypes of antibody. Mature B cells express antigen specific surface bound IgM and IgD immunoglobulin, or B cell receptors (BCR) (Le Bien *et al* 1998). The binding of specific antigen to the BCR activates the B cell leading to the proliferation and differentiation of a clonotypic population of antigen secreting B lymphocytes. These progeny cells produce antibodies of an array of heavy chain isotypes, with the specificity of the progenitor cell BCR, and thus mediate B cell effector function. This section describes the intracellular events that follow antigen ligation of the BCR, and couple to pathways which lead to B cell proliferation and antibody secretion (Le Bien *et al* 1998).

1.9.1 BCR STRUCTURE

The B cell receptor consists of an antigen binding membrane bound immunoglobulin component which is non covalently associated with a signal transducing/transporting, cytoplasmic substructure composed of disulfide linked dimers of immunoglobulin α (Igα)

and immunoglobulin β (Ig-β) (Campbell *et al* 1991). The Igα and Igβ chains of the BCR contain immunoglobulin tyrosine activation motifs which become phosphorylated upoon BCR ligation by the constitutively associated Src PTKs Fyn, Blk, lck and Lyn (Gold *et al* 1990) which leads to the recruitment of the PTK Syk (Lankester *et al* 1996). Evidence from co-capping and co-modulation studies identified a constitutive association between the BCR and a complex containing the CD19 receptor which is in turn complexed with the tetraspan web proteins Leu13/CD21/CD81 (Pesandot *et al* 1989). Thus, a role for CD19 in modulating BCR mediated signal transduction was proposed (see diagram 9).

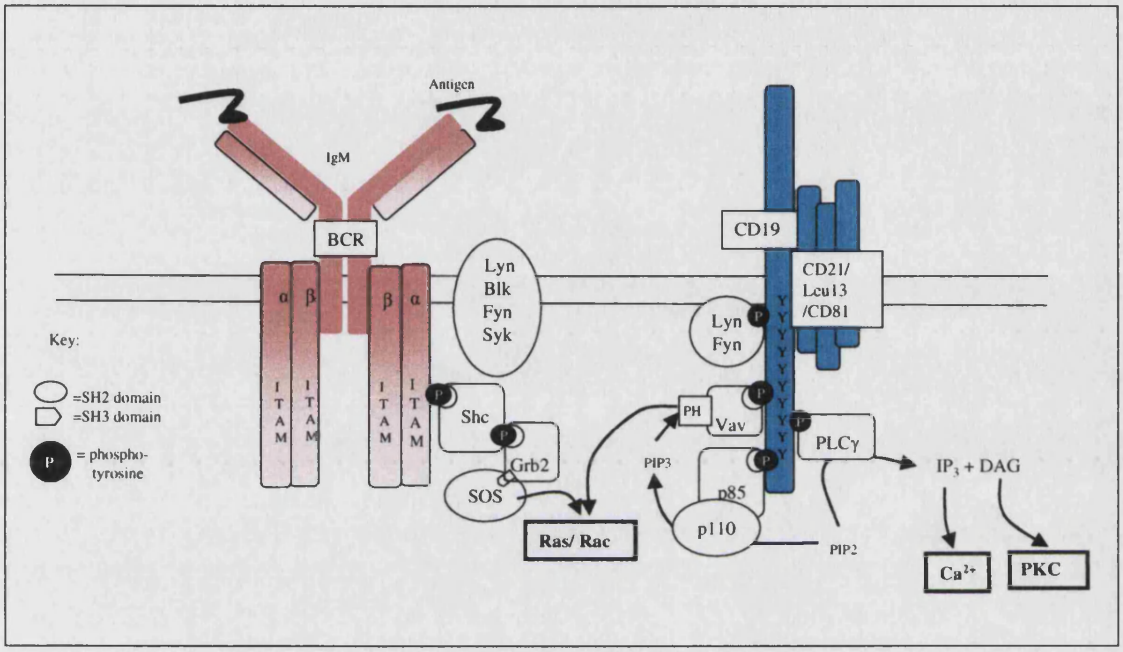


Diagram 9: BCR/CD19 Complex. Antigen ligation induces PTK activation and phosphorylation of the ITAMs of the BCR by Syk. CD19 becomes phosphorylated by constitutively associated PTKs, Lyn and Fyn, allowing the association of PLCγ, Vav, and p85. Association of Shc with the BCR Igα chain allows its phosphorylation, and association with the SH2 domain of Grb2, which recruits SOS to the membrane via its association with the SH3 domain of Grb2. The assembly of these protein complexes results in the mobilisation of intracellular calcium, activation of PKC, activation of Ras and Rac effector pathways and the recruitment of PH domain containing proteins to the membrane.

1.9.2 CD19

CD19 is a 95 kDa membrane bound glycoprotein that belongs to the Ig superfamily. It is expressed on B cells, at gradually increasing levels with maturation. Structurally CD19 has two immunoglobulin domains and an extensive cytoplasmic tail which comprises nine tyrosine motifs (Tedder *et al* 1989). CD19 is expressed prior to BCR expression and transmits signals throughout the different stages of B cell ontogeny. CD19 is phosphorylated in a ligation dependent manner by constitutively associated PTKs, *lyn* and

fyn (Uckun *et al* 1993). Tyrosine phosphorylated motifs within the CD19 intra-cytoplasmic tail act as docking sites for SH2 bearing molecules. The SH2 domain of Vav proto-oncogene product binds the Y^{393} EEP motif in the CD19 tail (Songyang *et al* 1994), and is thought to contribute to the phosphorylation of Mek1 and activation of the MAPK pathway in B cell pre-cursors (Weng *et al* 1994). CD19 bears two YXXM motifs (Tyr^{484}Gln - Asp-Met and Tyr^{515}-Glu-Asn-Met) (Zhou *et al* 1991), which have specificity for the SH2 domains of PI3K, and the presence of CD19/Vav/ PI3K complexes have been detected in B cell pre-cursors (Shanafelt *et al* 1995).

1.9.3 BCR LIGATION VERSUS BCR/CD19 COSTIMULATION

CD19 has been heavily implicated in the costimulation of BCR mediated signalling pathways (see diagram 9). Whilst BCR stimulation alone leads to elevated calcium mobilisation (Buhl *et al* 1998), and activation of ERK/MAPKs (Li *et al* 1997, Li *et al* 1998). Co-ligation of CD19 and the BCR synergistically enhances Map kinase activation (Li *et al* 1998) association and activation of PI3K (Tuveson *et al* 1993) and calcium mobilisation (Carter *et al* 1991). Co-ligation of the BCR and CD19 is thought to lower the threshold for B cell activation by two orders of magnitude (Dempsey *et al* 1996).

The *in vivo* significance of CD19/BCR co-stimulation has been illustrated in knock-out studies where it appears that CD19 is essential for germinal centre development, and maintenance of the B1 peritoneal subset of the B cell population. CD19 knockout mice exhibit normal B cell development, but impaired responses to antigens and reduced levels of circulating immunoglobulins (Ricket *et al* 1995, Engel *et al* 1995).

1.10 BCR EFFECTOR PATHWAYS

1.10.1 BCR MEDIATED MAP KINASE ACTIVATION

BCR ligation has been described to induce MAPK activation via several routes:

Firstly, BCR ligation results in the assembly of Grb2/ Shc/SOS complexes (see diagram 9) following the phosphorylation and recruitment of Shc to the Igα and Igβ chains of the BCR. This leads to the accumulation of active GTP-bound Ras (Saxton *et al* 1994, Harwood *et al* 1993) which can mediate the Raf /MEK /ERK MAPK pathway (Tordai *et al* 1994, and Li *et al* 1998). The existence of additional or more significant pathways coupling the BCR to MAPK activation was suggested following the observation that there is no great decrease in BCR induced MAPK activation in Grb2 deficient B cells (Harmer *et al* 1999). Thus a second pathway, is suggested to lead to MAPK activation downstream of

the BCR in which PKCα mediates Grb2 independent phosphorylation of Raf (Kolch et 1993).

A third route by which MAPK activation could be achieved following BCR ligation involves Vav (Gulbins *et al* 1997). VAV acts as a GTP exchange factor for Rac /CDC42 family GTP binding proteins which can augment the activity of PAK and MEK1 kinase (MEKK). PAK and MEKK elicit phosphorylation of MEK 1, which synergises with Raf mediated activation of MEK1 to upregulate ERK activity (Yan *et al* 1994, Lange-Carer *et al* 1993).

The exact pathway/s by which the enhanced phosphorylation of ERK arises following simultaneous CD19 and BCR ligation are unclear, however it is not accompanied by enhanced Ras or Raf activation, and is calcium independent and insensitive to PKC inhibition. Additionally, although reports have described Vav elicits synergistic activation of the MAPK cascade following BCR and CD19 co-ligation (Li et 1997), this has been disputed by studies performed in Vav mutant mice (O'Rourke *et al* 1998).

1.10.2 BCR MEDIATED ACTIVATION OF PI3K EFFECTORS

BCR ligation results in the accumulation of the PI3K D3 phosphoinositide products $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Gold *et al* 1994). PI3K activation is considered to occur upon co-association with the conserved tyrosine residues within the YXXM motif of CD19 which is phosphorylated in response to BCR ligation (Carter *et al* 1997, Tuveson *et al* 1993). Mutational studies have demonstrated that disruption of Y^{482} of the CD19 tail abrogates the PI3K/CD19 co-association and abrogates PI3K activity (Carter *et al* 1997). It has been reported that p85 may also interact with the BCR Ig α and Igβ chains (Clark 1992), and that PI3K can be activated *in vitro* by Fyn and Lyn binding via their SH3 domains to proline rich regions of p85 (Chalupny *et al* 1995). Evidence suggests however that PI3K is activated predominantly via CD19, as CD19 -/- B cells display a similar deficiency in BCR mediated signalling to that observed following wortmannin treatment of wild type B cells (Buhl *et al* 1997).

Studies in which the p85α adaptor subunit of PI3K is disrupted, further implicate PI3K in pathways leading to B cell development, survival, and proliferation. Lymphocytes from p85-/- deficient mice display severely impaired B cell development and have a reduced number of mature B cells in the peripherary, reduced serum Ig levels and a complete

absence of the $CD5^+$ B1 sub-population of B cells (Suzuki *et al* 1999 and Fruman *et al* 1999).

BCR/PI3K/BTK/ ACTIVATION

The B cell Tec family kinase (See diagram 2) BTK has been implicated as a BCR activated PI3K target that mediates calcium mobilisation in B cells through the phosphorylation of PLCγ2 (Fluckiger *et al* 1998, Buhl *et al* 1999). The membrane localisation of BTK is directed, in response to BCR ligation, by the association of $PI(3,4,5)P_3$ with the BTK PH domain. This mediates the approximation of BTK with membrane localised Src and Syk PTK activity (Varnai *et al* 1999).

Evidence that BTK activation lies downstream of CD19 activated PI3K, may be drawn from studies in which BTK activation was abrogated in cells carrying a targeted disruption of the CD19-PI3K SH2 binding motif (Buhl *et al* 1999). Accordingly, in a CD19 deficient plasmacytoma cell line, and wortmannin treated wild type cells, BTK activation was drastically reduced

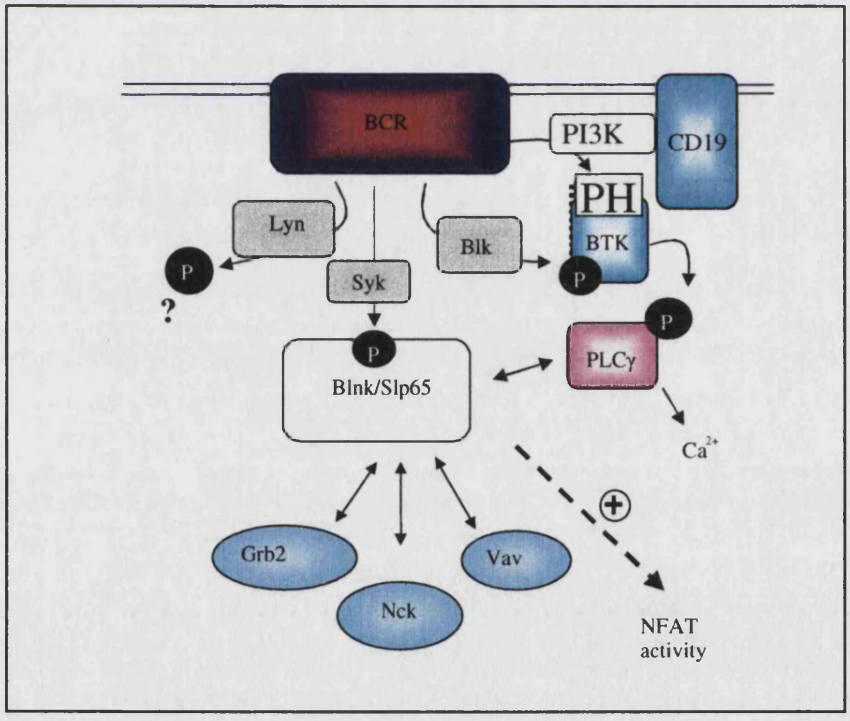


Diagram 10: BLNK the B cell homologue of SLP76. In B cells Blnk is a structural homolog of SLP76. It shares the capacity of SLP76 to associate withGrb2 Vav and PLCγ. Blnk/Slp65 is an additional target for Syk PTK activity, and following its phosphorylation, Blnk can act as a cytosolic adaptor protein, recruiting PLCγ. BCR ligation also leads the phosphorylation of Btk by Blk. Btk is recruited to the membrane and into the vicinity of Blk via the association of $PI(3,4,5)P_3$ with its PH domain.

in response to BCR ligation (Buhl *et al* 1999). Further, the similarity between impaired BCR triggered calcium responses, observed in cells from Xid mice, CD19-/- deficient cell lines, Y^{484} Y^{515}CD19 mutant cell lines (Buhl *et al* 1999) and p85α-/- mice (Suzuki *et al* 1999, Fruman *et al* 1999) supports the CD19 and PI3K dependency of BTK activation. BTK has been shown to bind the BLNK protein (see diagram 10), which is the B cell homologue of SLP-76, via the BTK SH2 domain and mutation of BTK SH2 domains has been shown to inhibit calcium mobilisation (Takata *et al* 1996). Additionally mutation of BLNK has been demonstrated to prevent PLCγ activation, calcium mobilisation and MAPK activation (Ishiai *et al* 1999). Thus it has been proposed that upon BCR ligation BLNK is phosphorylated by Syk, whereupon it is able to mediate an interaction between the SH2 domain of BTK, and PLCγ (Ishiai *et al* 1999).

The physiological importance of BTK in B cells is emphasised by B cells from Xid mice which carry a Cys-Arg mutation within the PH domain of BTK (Fukuda *et al* 1996) which causes impaired B cell development, high levels of BCR expression, and low serum Ig levels (Lindsberg *et al* 1991). In humans, a more expansive mutation, is the causative factor of the severe immunodeficieny, X-linked agamma-globulinaemia (XLA), and is characterised genetically by deletions and insertions in various BTK regions, including the SH3 domain (Zhu *et al* 1994). The aetiology of this deficiency is defined by arrest at early stages of B cell development, drastically reduced numbers of peripheral B cells which express high levels of surface IgM, and decreased circulating serum Ig levels (Vihinen *et al* 1997).

BCR/PI3K/PKB PATHWAY

PKB has been shown to be activated following BCR ligation in murine and human B cells (Gold *et al* 1999). PKB activation mediated via the BCR was shown, as in other systems (Cross *et al* 1995), to lead to the phosphorylation of GSK3 Astoul *et al* 1999) which correlated with the inactivation of GSK-3 enzymatic activity demonstrated in a further study (Gold *et al* 1999). Furthermore, using DT40 PTK deficient cell lines, it was shown that both *lyn* and *syk* kinase activity are required for PKB activation in B cells (Gold *et al* 1999). Furthermore it was demonstrated that BCR ligation, mediates the rapid and transient tranclocation of PKB to the membrane, whilst the activation of PKB kinase activity and PKB mediated phosphorylation of its downstream effector GSK3, are sustained for over one hour (Astoul *et al* 1999). Interestingly, constitutively active PI3K was shown to be sufficient to induce the transient recruitment of PKB from the nucleus and cytosol to the membrane, and sustain PKBs' enzymatic activation (Astoul *et al* 1999).

1.11 FCγRIIB MEDIATED INHIBITION OF B LYMPHOCYTE ACTIVATION

1.11.1 STRUCTURE / FUNCTION OF THE FCγRIIB ITIM

The FCγRIIB receptor is the low affinity inhibitory receptor for the immunoglobulin G constant region. The FCγRIIB bears a single immunoglobulin inhibitory tyrosine based motif (ITIM). This is a conserved 13 amino acid motif which is necessary and sufficient for inhibitory signalling in B cells. Within the ITIM the amino acid sequence: tyrosine-serine-leucine -leucine (Y^{309}SLL) is phosphorylated upon BCR / FCγRIIBco-ligation (Muta *et al* 1994, Kiener *et al* 1997). Although only one form of FCγRIIB receptor has been identified in mice, humans express two isforms, the FCγRIIB1 and the FCγRIIB2 which has a deletion of 19 amino acids between the ITIM and the trans-membrane region (Sarmay *et al* 1997).

Under physiological conditions the FCγRIIB mediates low affinity association with IgG FC regions. At high serum Ig levels a single IgG molecule can co-ligate the FCγRIIB and the BCR, via the IgG FC region and antigenic epitopes respectively (Morgan *et al* 1978). In this manner signalling through a variety of receptors including the BCR, and TCR, FCRI and FCRIII is inhibited (Daeron *et al* 1995). Targeted disruption of the FCγRIIB receptor showed its essential function in controlling anaphylactic responses in mice, and preventing autoimmunity and hyperglobulinaemia (Takai *et al* 1996). Co-crosslinking the BCR with the FCγRIIB generates an inhibitory signal which abrogates proliferation and differentiation of B lymphocytes. The underlying mechanisms which lead to the inhibition of B cell signalling are not fully understood. A summary of the biochemical events that follow BCR/FCγRIIB co-ligation are outlined in the following sections:

1.11.2 ASSOCIATION OF SH2 CONTAINING MOLECULES WITH THE FCγRIIB ITIM

In murine B cells co-ligation of the FCγRIIB with the BCR leads to the co-association and and activation of the lipid and protein phosphatases SHIP, SHP1 and SHP2 with the phosphorylated ITIM (Sarmay *et al* 1997) (see diagram 11). The avidity of their interactions decreases thus: SHIP> SHP2> SHP1 (D'Ambrosio *et al* 1995). Assembly of these proteins at the membrane brings them into proximity with the activating receptor and its associated signalling complexes, upon which the ITIM binding molecules presumably exert their effects. In other murine cell types the FCγRIIB ITIM has different specificities,

eg; in bone marrow derived mast cells FCγRIIB receptors co-aggregated with FCεR1 selectively binds to SHIP (Fong *et al* 1996).

SHP1

SHP1 is a cytosolic protein tyrosine phosphatase with two N terminal SH2 domains, a

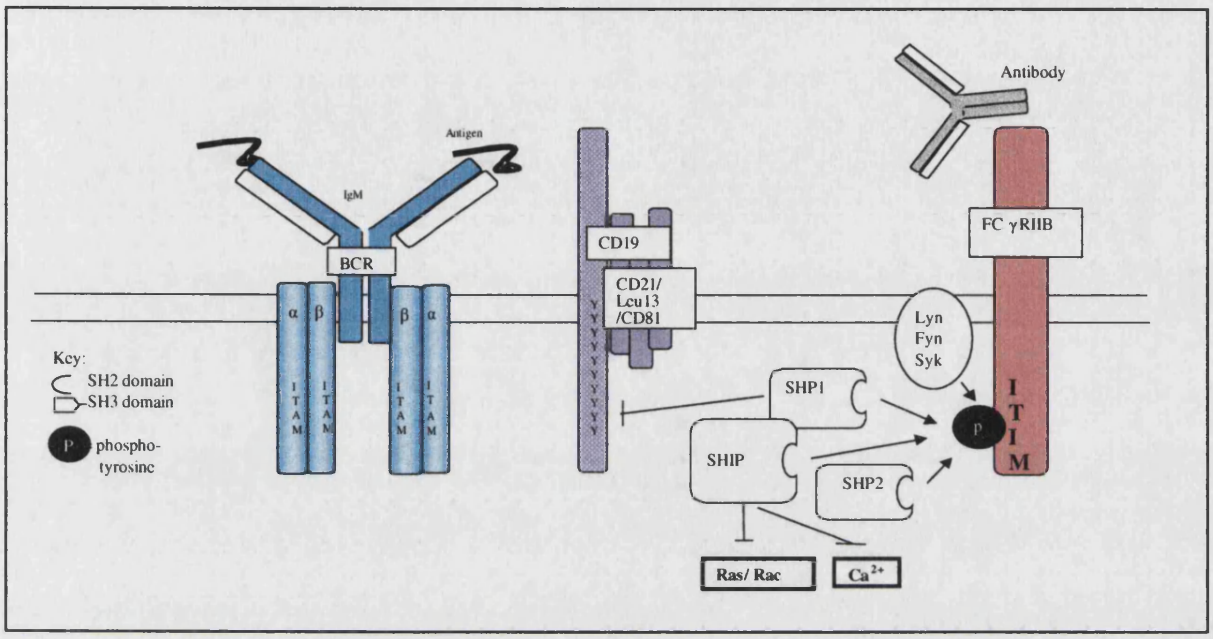


Diagram 11: BCR/CD19 Coligation with the FCγRIIB. Simultaneous ligation of the BCR with antigen and the FCγRIIB ligation with antibody leads to the phosphorylation of the FCγRIIB ITIM, and the subsequent recruitment of SHIP SHP1 and SHP2 to the ITIM. SHIP is implicated in the abrogation of MAPK signalling pathways and calcium mobilisation whilst SHP1 is thought to mediate de-phosphorylataion of CD19. A role for SHP2 in inhibitory signalling cascades still remains to be defined.

phosphatase domain and C terminal tyrosine phosphorylation sites (Shen *et al* 1991). SHP1 phosphatase activity is upregulated upon its association with the phosphorylated ITIM (D'Ambrosio *et al* 1995). Indeed the de-phosphorylation of CD19 was thought to be attributable to the phosphatase activty of SHP1 (Kiener *et al* 1997). Mutations in the SHP1 gene, carried by motheaten lead to aberrant FCγRIIB inhibiton of BCR signalling (D'Ambrosio *et al* 1995, Olcese *et al* 1996). Conflicting evidence is presented by studies which showed that SHP1 was dispensable for FCγRIIB mediated signalling, as in SHP1 deficient DT40 cells normal calcium mobilisation and NFAT translocation to the nucleus was observed (Ono *et al* 1997).

SHP2

SHP2 is an SH2 domain containing phospho-tyrosine phosphatase (PTPase) which displays two N terminal SH2 domains, and a C terminal catalytic domain. SHP2 is

ubiquitously expressed (Feng *et al* 1993, Freeman *et al* 1992), and multiple studies in haemopoietic cells have shown that SHP2 is subject to tyrosine phosphorylation in response to multiple growth factors and cytokines (Gadina *et al* 1998, Welham *et al* 1994). SHP2 can physically associate, via its SH2 domains with growth factor and cytokine receptors (Yamauchi *et al* 1995, Lechleider *et al* 1993, Tauchi *et al* 1996, Bone *et al* 1997), IRS-1 (Kuhne *et al* 1993), CrkL (Chin *et al* 1997), the PI3K p85 adaptor sub-unit (Welham *et al* 1994, Craddock and Welham 1997, Zhang *et al* 1999) and Gab2 (Gu *et al* 1998). Tyrosine phosphorylated SHP2 has also been reported to associate with Grb-2 and SHIP (Bennett *et al* 1994, Welham *et al* 1994, Liu *et al* 1996).

Efforts to identify PTPase substrates for SHP2 have putatively indicated that Gab2 (Craddock and Welham 1997, Zhang *et al* 1998, Gadina *et al* 1999, Frearson *et al* 1998) Gab1 (Holgado Madruga *et al* 1996, Nakamura *et al* 1998), and SIRP (Stofega *et al* 1998, Kharitonenkov *et al* 1997) may be subject to PTPase activity directed by SHP2. SHP2 has been implicated in immediate early, and late signal transduction which elicits progression of the cell cycle (Bennett *et al* 1996), in cell survival (Pazdrak *et al* 1997) and in mitogenesis (Xiao *et al* 1994). SHP2 is thought to mediate enhanced Ras activation leading to MAPK activation via its interactions with Grb2, and Gab2 (Noguchi *et al* 1994, Bennett *et al* 1996, Li *et al* 1994, Gu *et al* 1998, Nishida *et al* 1999).

Relatively little is understood of SHP2 function in mediating FCγRIIB inhibitory signals. SHP2's stimulatory role in other systems, suggested that it may mediate a positive role in B cells (Tamir *et al* 2000). However studies which used decoy proteins to block SHP2s association with the ITIM demonstrated an inhibitory role for SHP2 in ERK phosphorylation and calcium mobilisation (Nakamura *et al* 2000). Efforts to identify proteins with which SHP2 may associate in B cells have indicated an association between SHP2 and Gab1 which is enhanced following BCR ligation (Nakamura *et al* 1998, Nishida *et al* 1999).

1.12 FCγRIIB INDUCED NEGATIVE REGULATION OF PI3K ACTIVITY

1.12.1 FCγRIIB AND SHIP- INHIBITION OF PIP3 ACCUMULATION

Upon FCγRIIB co-ligation SHIP is recruited via its SH2 domain to the phosphorylated tyrosine ^{309} residue in the ITIM (Muta *et al* 1994). This association leads to the rapid and substantial tyrosine phosphorylation of the lipid polyphosphate 5'-phosphatase, SHIP (Chacko *et al* 1996). SHIP phosphorylation correlates with an upregulation of SHIPs' catalytic activity (Sarmay *et al* 1997) and its subsequent recruitment of tyrosine

phosphorylated Shc (Liu *et al* 1997). SHIP has been implicated in negative signalling following BCR /FCγRIIB co-ligation through its enzymatic degradation of PI(3,4,5)P_3, the PI3K metabolic product to PI(3,4)P_2 (see diagram 12) and therefore SHIP could be responsible for the reduced accumulation of this lipid under negative signalling conditions (Scharenberg *et al* 1998).

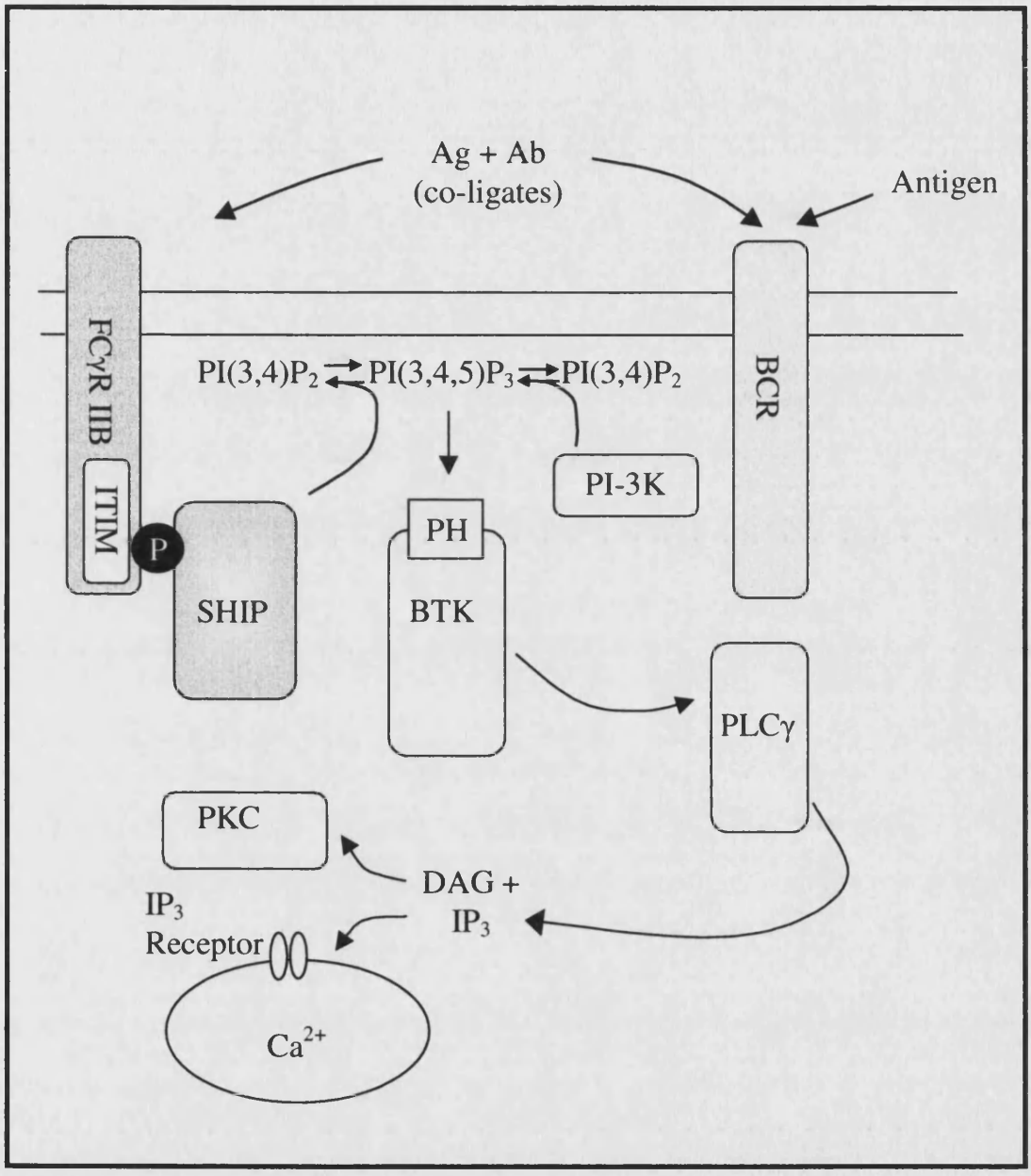


Diagram 12: Enzymatic function of SHIP in calcium signalling.

Co-ligation of the BCR with the FCγRIIB, via simultaneous occupancy by antigen and antibody respectively, allows the phosphorylation of the FCγRIIB ITIM, which subsequently recruits, and elicits the phosphorylation of SHIP. SHIP catalytic activity acts to reverse the accumulation of PI (3,4,5)P_3, leading to the production of PI(3,4)$_2$. The lack of PI(3,4,5)P_3 at the cell membrane prevents the recruitment of Btk and significantly attenuates calcium mobilisation.

Multiple reports argue that SHIP is the predominant inhibitory effector molecule that mediates FCγRIIB's modulation of BCR signalling. One such study blocked SHIP binding

to the ITIM using SHIP decoy proteins and demonstrated the abrogation of SHIP's recruitment of Shc, and inhibition of PI(3,4,5)P_3 accumulation, calcium mobilisation, and Erk activation (Nakamura et 2000). Furthermore, in a SHIP deficient DT40 chicken B cell line it was shown that SHIP is an absolute requirement for FCγRIIB mediated inhibition of calcium signalling (Ono *et al* 1996 and Hashimoto *et al* 1999), and the calcium dependent re-localisation of NFAT to the nucleus. In addition mutation of the SH2 binding tyrosine residue of the ITIM or mutation of SHIP's catalytic activity can also prevent inhibition of BCR mediated pathways (Aman *et al* 2000). SHIP has also been implicated in apoptosis: SHIP recruitment attenuates a proapoptotic signal initiated by FCγRIIB BCR coligation (Ono *et al* 1996). A later study demonstrated that FCγRIIB can signal independently of BCR coligation to directly mediate an apoptotic response, requiring only an intact transmembrane domain. Failure to recruit SHIP, either by deletion of SHIP or mutation of FCγRIIB, results in enhanced FCγRIIB-triggered apoptosis (Pearse *et al* 1999).

1.12.2 FCγRIIB MODULATION OF BCR / PI3K / PKB PATHWAYS

FCγRIIB co-ligation with the BCR negatively regulates PKB activation, and this is thought to be mediated through SHIP by the hydrolysis of PI(3,4,5)P_3 (Astoul *et al* 1999). Accordingly, FCγRIIB co-ligation inhibits the membrane localisation and enzymatic activity of PKB (Astoul *et al* 1999).

Studies in SHIP deficient Avian B cells showed that SHIP is essential for FCγRIIB co-ligation induced inhibition of PKB activation, and GSK3 inhibition (Jacob *et al* 1999). Similarly in FCγRIIB deficient cells, which could not activate SHIP, impaired negative regulation of PKB was evident (Aman *et al* 1998), further indicating that SHIP is an essential component in the negative regulation of PKB.

1.12.3 FCγRIIB MODULATION OF PI3K / BTK AND PLCγ

Co-ligation of the BCR and FCγRIIB abrogates PLCγ phosphorylation which in turn leads to the inhibition of IP_3 production (Bijsterbosch *et al* 1985, Sarkar *et al* 1996) and the sustained extracellular influx of calcium that occurs in response to BCR ligation (Diegel *et al* 1994). The degradation of PI(3,4,5)P_3 by SHIP in response to FCγRIIB ligation is thought to prevent recruitment of the PH domain containing protein BTK to the membrane where it is further activated by PTK activity (Scharenberg *et al* 1998). The abrogation of BTK phosphorylation of PLCγ (See diagram 12) (Scharenberg *et al* 1998), and the subsequent inhibition of PI(4,5)P_2 hydrolysis mediated by PLCγ, may be responsible for the diminished calcium responses observed following BCR/FCγRIIB co-ligation (Falasca

et al and Scharenberg *et al* 1998, Ono *et al* 1996). Accordingly, overexpression of a BTK membrane associated chimaera can reverse the inhibitory effects of FCγRIIB co-ligation; membrane expression of SHIP, or wortmannin inhibition of PI3K on BCR triggered calcium signalling (Bolland *et al* 1998). In further support of a role for BTK in calcium mobilisation it was shown that deletion of SHIP increased the membrane localisation of BTK, via elevated PI(3,4,5)P_3 accumulation (Bolland *et al* 1998).

1.13 FCγRIIB INHIBITION OF MAPK CASCADE

1.13.1 FCγRIIB / SHIP / SHC

FCγRIIB results in the inhibition of Ras (Sarmay *et al* 1996), Raf-1 induction (Moodie *et al* 1994) and Erk activity (Campbell *et al* 1995). SHIP is thought to compete with Grb2 for binding to Shc and thus could be responsible for the decrease in GTP bound Ras observed on FCγRIIB co-aggregation. (Tridandipani *et al* 1997). The recruitment of Shc by SHIP is mediated by a bidentate interaction which occurs between i) the NpxY motif of SHIP and the PTB domain of tyrosine phosphorylated Shc, **and** ii) the SHIP SH2 domain binding the doubly phosphorylated residuesY^{239} and Y^{240} of Shc ((Harmer *et al* 1999, Liu *et al* 1997, Pradhan *et al* 1997). SHIP knockout mice lend support to a role for SHIP in the abrogation of MAPK as in the absence of SHIP, FCγRIIB co-aggregation does not inhibit ERK phosphorylation (Liu *et al* 1998).

1.13.2 FCγRIIB / P62 DOK / RAS GAP

A further route has been proposed to inhibit MAPK activation upon BCR/FCγRIIB co-ligation. In addition to regulation by GEF's the inactivation of the intrinsic GTPase activity of Ras is thought to be essential for its full activation and maintenance of a GTP bound state. Ras GAP is a Ras GTPase which has been shown to enhances the GTPase activity of Ras causing the hydrolysis of GTP to GDP (Lazarus *et al* 1998). Upon BCR ligation Ras Gap is thought to bind the tyrosine phosphorylated adaptor protein p62-Dok-1 via a PTB domain mediated interaction (Yamanashi *et al* 1997). FCγRIIB co-ligation further enhances the phosphorylation of p62 which enhances its interaction with Ras Gap via aa 260-482 in the p62 Dok-1 tyrosine rich motif. In addition p62 Dok-1 and SHIP co-associate via the p62 Dok-1 PTB domain (aa 1-259) which presumably binds one of SHIP's NPXY motifs (Tamir *et al* 2000). Phosphorylation of p62 Dok-1 is dependent upon both SHIP and an intact ITIM, suggesting that SHIP mediates p62 Dok-1 association with the ITIM and thus proximity with FCγRIIB activated PTKs. The inhibition of Erk activity correlates with Ras Gap p62 association but not SHIP p62. This is thought to occur independently of Shc as Shc and SHIP are thought to exist in distinct complex to p62 Dok-

1 and Ship (Tamir *et al* 2000). B cells from p62 Knock out mice show impaired FCγRIIB mediated inhibition of ERK activation which further supports a role for p62 Dok-1 in regulating Ras activity via Ras Gap.

1.14 AIMS AND OBJECTIVES

The delineation of mechanisms, which regulate signal transduction pathways in T and B lymphocytes, is essential in order that immunotherapeutic strategies leading to manipulation of the immune system can be designed. PI3K has been widely implicated in the biochemical events which characterise T and B lymphocyte activation(Reif *et al* 1996, Klippel *et al* 1997, Tuveson *et al* 1993). However, data that has questioned the significance of PI3K in T lymphocytes has arisen from studies in which p85 deficient mice were generated. Whilst p85 deficient B cells exhibit profoundly heightened immune responses and basal levels of activation, T cells from these mice were shown to be phenotypically normal (Suzuki *et al* 1999, Fruhman *et al* 1999). Similarly, further studies which examined the regulation of PI3K lipid product accumulation in SHIP deficient mice again identified a normal signalling phenotype in T cells, whilst B cell responses were perturbed (Liu *et al* 1999, Helgason *et al* 1998).

The primary objective of this study was to further investigate the intracellular pathways that regulate PI3K activity in T and B lymphocytes downstream of CD28 and the BCR.
The aims of this study were four fold:

1) To examine the differential recruitment of PI3K catalytic isoforms p110α, p110β, and p110δ to the T cell costimulatory receptor CD28. The identification of p110δ, a novel isoform of PI3K which displays a restricted tissue distribution, in haemopoietic cell lineages, poses many questions as to the role of this isoform in Lymphocytes.
2) To investigate the regulation of the SH2 containing inositol 5'-(poly)phosphatase (SHIP) mediated by CD28 and CTLA4 initiated signalling cascades in T lymphocytes.
3) In an effort to explain the data from studies which have claimed that PI3K does not play a significant role in the leukaemic T cell model, Jurkat, this study will examine the regulation of 3'-phosphoinositdes accumulation in this cell line.

4) Using the A20 B cell lymphoma cell line this study will examine the nature of the FCγRIIB signalling cascade with regard to its inhibition of BCR mediated PI3K dependent signalling cascades.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PLASMIDS PROTEINS

PLASMID/PROTEIN	SOURCE/COMMENT
pGST-SH2-SHIP	Generous gift from Mark Coggeshall, Ohio state University USA. GST tagged SH2 domain of SHIP expressed in pGEX vector.
pGST-NSH2-p85	Generous gift from Dr Melanie Welham, University of Bath, UK. GST tagged N terminal SH2 domain of p85 expressed in pGEX vector.
pGST-CSH2-p85	Generous gift from Dr Melanie Welham, University of Bath, UK. GST tagged C terminal SH2 domain of p85 expressed in pGEX vector.
pGFP-PKB	Generous gift from Julian Downward, ICRF, London, UK. GFP tagged PKB expressed in pEGFP vector.
pGFP- R^{25}CPKB	Generous gift from Julian Downward ICRF, London, UK GFP tagged PKB PH R^{25}C mutant expressed in pEGFP vector .
pGST-SH2-SHP2 (FL)	Generous gift from Dr Helen Wheadon, University of Bath, UK. GST tagged SHP2 N and C terminal SH2 domains expressed in pGEX2T vector .
pGST-SH2-SHP2 (C)	Generous gift from Dr Helen Wheadon, University of Bath, UK. GST tagged SHP2 C terminal SH2 domain expressed in pGEX2T vector.
pGST-SH2-SHP2 (N)	Generous gift from Dr Helen Wheadon, Bath University, UK. GST tagged SHP2 N terminal SH2 domain expressed in pGEX2T vector.
prCD2- SHIP	Generous gift from Doreen Cantrell, ICRF, London, UK. rat CD2 transmembrane domain with SHIP catalytic core (364-825aa) expressed in pEF BOS.

prCD2- C^{671}A SHIP	**Generous gift from Doreen Cantrell ICRF, London, UK., CD2 transmembrane domain with point mutant C/A671 SHIP catalytic core (364-825 aa) expressed in pEF BOS.**
p85/p110α **p85/p110β** **p85/p110δ** **p85/p110Pδ**	**Geneorous gift from Bart Vanhaesebroeck, Ludwig institute London. Recombinant proteins comprising bovine p85 coupled to human p110 subunits. Expressed in SF9 cells and purified using Actigel beads coated with PDGF receptor peptide.**

2.1.2 ANTIBODIES

ANTIBODY	SPECIES	MONO/ POLYCLONAL	SOURCE
F(ab')CT29 Anti-CTLA4	**Mouse**	**monoclonal**	**Carl June, Naval medical research institute, Bethesda, USA.**
F(ab')2 anti mouse IgG	**Rabbit**	**polyclonal**	**Zymed, USA**
anti mouse IgG	**Rabbit**	**polyclonal**	**Zymed, USA.**
anti-CTA4 (3D6)	**Mouse**	**monoclonal**	**Carl June, Naval medical research institute, Bethesda**
anti-CTLA4 (CT29)	**Mouse**	**monoclonal**	**Carl June Naval medical research institute, Bethesda**
anti-CD28 (9.3)	**Mouse**	**monoclonal**	**Carl June, Naval medical research institute, Bethesda**
anti-p85α	**Mouse**	**monoclonal**	**Doreen Cantrell, ICRF, London.**

anti-p110δ	Rabbit	polyclonal	Bart vanhaesebroeck, ludwig institute, london
anti-p110β	Rabbit	polyclonal	Santa Cruz
anti-p110α	Goat	polyclonal	Santa Cruz
anti-SHIP	Goat	polyclonal	Santa Cruz
anti-SHIP (aa 874-941)	Rabbit	polyclonal	Mark Coggeshall, Ohio state university, OH.
anti-hCD3 UCHT1	Mouse	monoclonal	Generous gift from Doreen Cantrell, ICRF, London, UK.
anti-mCD3 2C11	Mouse	monoclonal	Daniel Olive, INSERM, Marseille.
anti-mFCγRIII/II mAb	Rabbit	polyclonal	Pharmingen San Diego, USA.
purified mIgG2a	Mouse	monoclonal	Sigma, Poole, Dorset
anti-CTLA4 (CT29)	Mouse	monoclonal	Carl June, Naval medical research institute, Bethesda
anti-CTLA4 (3D6)	Mouse	monoclonal	Carl June, Naval medical research institute, Bethesda
anti-ratCD2 (OX34)	Mouse	monoclonal	Doreen Cantrell, ICRF, London,UK.
anti-phospho ERK 1/2	Rabbit	polyclonal	NEB, New England, USA.
anti-ERK1/2	Rabbit	polyclonal	NEB, New England, USA.
anti-phosphotyrosine (4G10)	Mouse	polyclonal	Upstate Biochemical Industries, NJ, USA.
anti-p62 DOK1	rabbit	monoclonal	Santa Cruz,
anti-Gab2	Rabbit	polyclonal	Upstate Biochemical Industries, NJ, USA
anti-SHP2	Rabbit	polyclonal	Santa Cruz,
anti Rabbit-FITC	Goat	polyclonal	Sigma, Poole UK.
anti mouse-FITC	Rabbit	polyclonal	Sigma, Poole, UK.

anti-GST	Mouse	monoclonal	Upstate Biochemical Industries
anti- CD19	Rat	monoclonal	Sigma, Poole, Dorset
anti-MHCDR abL243	Rabbit	polyclonal	Dave Sansom, Bath University Bath UK.
anti-mouse HRP	Rabbit	polyclonal	DAKO, Denmark.
anti-Goat HRP	Sheep	polyclonal	DAKO, Denmark.
anti-Rabbit-HRP	Sheep	polyclonal	DAKO, Denmark.
anti-CD14 (UCHM1)	Rabbit	polyclonal	Dave Sansom, Bath University, Bath UK.
anti-mouse IgG coated magnetic beads.	Sheep	polyclonal	Dynal, Merseyside,UK.

2.1.3 GENERAL REAGENTS

REAGENT	SOURCE
[^{32}P]-orthophosphoric acid 5 mCi/ml	NEN, Stevenage UK.
[^{32}P]γ-ATP 5 µCi/ml	NEN, Stevenage, UK
[^{3}H]- inositol [1,3,4,5]- tetrakisphosphate	NEN, Stevenage UK
2-Mercaptoethanol	Sigma,Poole UK.
3MM filter paper	Whatmann, UK
Acetone	BDH, Poole, UK.
Acrylamide	Biorad,
Agarose (Molecular Biology grade)	Sigma,Poole UK.
Ammonium phosphate	Sigma, Poole, Uk.
Ampicillin	Sigma,Poole UK.
Aprotinin	Sigma,Poole UK.
ATP	Sigma,Poole, UK.
Bovine Foetal calf serum (heat inactivated)	Gibco, BRL
BSA (Tissue culture grade)	Sigma,Poole UK.

Butanol	**BDH, Poole Uk.**
Calcium Chloride	**Fisher, Loughborough, UK.**
Chloroform	**BDH, Poole U.K.**
Cryovials	**NUNC, UK.**
Dabco	**Sigma, Poole, UK.**
DMEM with L Glu without Na Pyruvate, without Na bicarbonate	**Sigma, Poole, UK**
DMEM without Glutamine, with pyridoxine, with Na bicarbonate	**GIBCO BRL UK.**
DMSO	**Sigma,Poole UK.**
DNA 1KB ladder	**NEB**
ECL	**Amersham**
EDTA	**Sigma,Poole UK.**
Ethanol 99.1%	**BDH**
Ethidium bromide	**Sigma, Poole UK.**
Ethyl Formate,	**Fisher scientific, UK**
Flo-Scint IV liquid scintillant	**Canberra Packard, UK.**
Folsch lipids	**Sigma, Poole, UK**
Fungizone	**Gibco BRL**
Glacial Acetic Acid	**BDH, Poole, UK.**
Glucose	**Fisher, Loughborough, Uk**
Glutathione	**Sigma, Poole, UK**
Glutathione Sepharose	**Pharmacia, UK.**
Glycerol	**Sigma,Poole UK.**
β-Glycerophosphate	**Sigma, Poole, UK.**
HBSS (without calcium and magnesium)	**Gibco, Paisley UK.**
HCl	**BDH, Poole Uk.**
Heparin	**Fisons, loughborough UK**
HEPES (1M)	**Sigma, Poole, Dorset.**
HPLC column (SAX partisphere)	**Whatmann, UK.**
Iodine	**Sigma, Poole, UK**
Iodoacetamide	**Sigma, Poole, UK.**
Kanamycin	**Sigma,Poole UK.**

KCl	BDH, Poole, UK
KH_2PO_4	BDH Poole, UK
LB Agar	Sigma, Poole, UK.
LB broth (miller)	Sigma, Poole, UK.
Leupeptin	Sigma,Poole UK.
Lithium Chloride	Sigma,Poole UK.
Magnesium chloride	BDH Poole, UK
Manganese Chloride	BDH Poole, UK
Marvel	Supermarket
Methanol	BDH, Poole, UK
Methanol	BDH Poole, UK
Methylamine (25%-30%in dH_20)	Fisher, Loughborough, UK.
Methylamine (25%-30%in dH_20)	Fisher Loughborough, UK
Molecular Weight markers	Gibco, Paisley, UK.
MOPS	Sigma,Poole UK.
Na Cl	Sigma,Poole,UK.
$Na_2HPO_4.H_2O$	BDH,Poole UK.
NaF	Sigma, Poole UK.
Nitrocellulose	BDH, Poole, UK
NP40	BDH, Poole, UK
Paraformaldehyde	Sigma,Poole UK.
Pen/Strep	Gibco BRL, Paisley, UK.
Pepstatin	Sigma,Poole UK.
Petroleum ether (bp 40-60 °C)	BDH, Poole UK.
Phenyl Methyl Sulfonyl Flouride	Sigma,Poole UK.
Phosphatidyl inositol	Sigma, Poole, UK
Phosphatidyl serine	Sigma, Poole, UK
Phosphoric acid	Fisher, UK
PMA	Calbiochem, Nottingham UK.
Poly L lysine coated slides	BDH, Poole UK
Ponceau S	Sigma, Poole, Dorset.
Potassium Acetate	Sigma,Poole UK.

Propan-2-ol	**BDH Poole, UK**
Propan-I-ol	**BDH Poole, UK**
Protein A sepharose	**Pharmacia, Uk**
Protein G sepharose	**Sigma, Poole, Dorset**
Qiagen^{TM} endo –free DNA purification kit.	**Qiagen, UK**
Restriction enzymes(ECOR1, HindIII)	**Promega, UK**
RPMI 1640	**Gibco BRL, UK**
Rubidium chloride	**Fisher, UK**
SDS	**Sigma, Poole, UK**
SEB	**Fluka, Dorset, UK**
Sodium hydroxide	**BDH, Poole Uk**
Sodium Orthovanadate	**Sigma,Poole UK.**
Sucrose	**Fisher, UK**
TBAS	**Sigma, Poole, UK.**
TEMED	**Sigma, Poole, UK**
Terrific broth	**Sigma, Poole, Dorset.**
TLC plates (linear K	**Whatmann, Maistone, Kent.**
Tris.Hcl	**Sigma, Poole, UK**
Trypan Blue	**Sigma,Poole UK.**
Trypsin EDTA	**Gibco BRL, Paisley, UK.**
Tween	**Sigma, Poole, Dorset.**
Xomat film	**Kodak, Harrow, UK.**

2.1.4 GENERAL SOLUTIONS.

7.5% Gel (mini protean-gels x 1 mm thick)

(run at 100V for 1 hr)

30 % bis:acrylamide	2.1 mls
1M Tris.HCl pH 7.5	1.8 mls
H_2O	1.8 mls
10 % APS	15.0 µl
TEMED	3.5 µl
10 % SDS	25.0 µl

Gradient Gel (15 cm x 15 cm x 1.5 mm thick)

(run at 75V o/n)

	7%	17%
30% bis:acrylamide	3.5 mls	8.5 mls
1M Tris.HCl pH 7.5	7.6 mls	5.6 mls
H_2O	3.1 mls	---------
Sucrose	--------	1.59 g
10 % APS	50 µl	50 µl
TEMED	10 µl	10 µl
10 % SDS	75 µl	75 µl

Stacking Gel

30% bis:acrylamide	1.6 mls
H_2O	7.6 mls
Upper Buffer	3.1 mls
10 % APS	50 µl
TEMED	10 µl

Upper Buffer

Tris pH 6.8	0.5 M
SDS	0.4%

PBS (1X)- for western blotting

Na_2HPO_4	80 mM
$NaH_2PO_4.12\ H_2O$	20 mM
NaCl,	100 mM
pH 7.5	

Running Buffer –SDS (5L 10X)

Tris.HCl	25 mM
glycine	192mM
SDS	0.01%

Stripping buffer

Tris.HCl pH6.8	62.5 mM
dH_2O	48.8 mls
SDS	2 %
2- mercaptoethanol.	100 mM

SDS Laemmli sample buffer

Glycerol	10%
SDS	0.4%
Tris HCl pH 6.8	200 mM
2-Mercaptoethanol	5%
Bromophenol blue	To colour

Semi Dry Transfer Buffer

Glycine	39mM
Tris base	48 mM
SDS	0.0375%
Methanol	20%

2.2 METHODS

2.2.1 MOLECULAR BIOLOGY

PREPARATION OF COMPETENT CELLS

DH5 α E.Coli were picked from a single colony and streaked onto a pre-dried LB agar plate. After incubation at 37 ^{0}C overnight a single colony was picked and grown in 5 ml LB in an orbital shaking incubator at 37 ^{o}C overnight. Subsequently 1 ml of this culture was diluted in 100 ml of LB and further cultured until the optical density at 550 nm read 0.48. The culture was then immersed in ice to cool and then pelleted by centrifugation at 6,000 r.p.m in 50 ml tubes, for 5 minutes at 4 ^{o}C. Cells were then re-suspended in 40 mls of Tfb1 (30 mM KCl, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) Glycerol, pHd to 5.8 with glacial acetic acid), and incubated on ice at 4 ^{o}C. Cells were then resuspended in 4 mls of TfbII (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% (v/v) glycerol) and incubated on ice for 15 minutes. After 15 minutes the competent cells were aliquoted in 200 µl aliquots and snap frozen in a dry ice ethanol bath, prior to long term storage at –80 ^{o}C.

TRANSFORMATION

Plasmid DNA was transformed into competent DH5α via the heat shock method. 1 µg of stock DNA was mixed via gentle vortexing with 100 µl of competent cells which had been thawed on ice, and the mixture incubated on ice for 30 minutes. Cells were then placed at 42 ^{o}C for 90 seconds, and then diluted 1:10 in pre-warmed LB. The cells were placed at 37 ^{o}C for 30 minutes, and then spread onto a pre-dried agar plate containing ampicillin at 0.1 mg per ml, or, in the case of GFP-PH PKB and GFP-PH(618)-PKB, kanamycin at 50 ng per ml. The plate was allowed tor dry before inverting and growing at 37 ^{O}C overnight.

SMALL SCALE PLASMID PREPARATION

To verify the identity of plasmid DNA prior to large scale preparation a single colony was picked and placed in 3mls of LB with the apropriate antibiotic (As above) and grown overnight. 1.5 mls of the culture were pelleted and resuspended by vortexing in 100 µl solution I (200 mM Tris.HCl pH 8 , 50 mM glucose, 10 mM EDTA). The remaining culture was stored at 4 ^{o} for further use. Cells were lysed

through addition of 200 µl of solution II (0.2 N NaOH and 1 % SDS), and the plasmid DNA purified from the cell debris though addition of 150 µl ice cold solution III (60 mls 5M KOAc 11.5 mls glacial acetic acid 28.5 mls H_2O). Samples were incubated on ice for ten minutes prior to centrifugation. Supernatants were harvested and nucleic acids were subsequently precipitated with 0.7 vols room temperature isopropanol. Nucleic acid was pelleted via centrifugation at 13,000 r.p.m for 15 minutes and pellets were washed with 70 % ethanol, and subsequently air dried. Finally the nucleic acids were resuspended in 200-100 µl dH_2O, and stored at –20 °C. Concentration of DNA was determined crudely via measurement of optical density at 260 nm.

RESTRICTION ENZYME DIGESTION

DNA from small scale plasmid preparations was subject to confirmatory digestion using at least two appropriate restriction enzymes. 1 µl of DNA was cleaved using 0.5 Units (0.5 µl) of each restriction enzyme, 1µl 10X restriction buffer, 1µl 10X BSA in a total volume of 10 µl. Where larger volume digests were performed, volumes were adjusted accordingly, ensuring that the total volume of enzyme added did not exceed more than 10 % of the final digest volume.

LARGE SCALE PLASMID PREPARATION

Following confirmation that the chosen clone contained the correct plasmid, 1.0 ml of the retained mini culture was used to innoculate 500 ml of LB supplemented with the appropriate antibiotic. After incubation overnight at 37 °C, endotoxin free DNA was prepared using Qiagen™ Endo-Free plasmid preparation kit.

PREPARATION OF GST FUSION PROTIENS

A single bacterial colony was innoculated into 10 mls 2x YT supplemented with 50 µg per ml ampicillin and grown o/n at 37 °C. The overnight culture was then used to innoculate 500 mls 2xYT supplemented with ampicillin as before. The culture was grown at 37 °C until O.D = 0.6-0.8 and then induced with 100mM IPTG and incubated at 27 °C o/n.

Cells were then pelleted and washed in fusion protein lysis buffer and resedimented before resuspension in 15 mls of ice cold lysis buffer (10 mg/ml aprotinin, 20 mg/ml leupeptin, 2.8 mg/ml pepstatin, 80 mg/ml PMSF, 10 mg/ml Lysozyme, 10 mM 2-ME). The resuspended cells were incubated at 25 ^{o}C for 10 minutes and lysis was achieved by freeze / thawing on dry ice / 37 ^{o}C water bath x 3. Nucleic acids were then digested by the addition of 20 mM $MgCl_2$ and 0.14 mg Dnase1 and incubation for a further 15 minutes, with agitation, at 25 ^{o}C. The reaction is stopped with EDTA (20 mM) and 630 µl 10% NP40 was added prior to incubation at 25 ^{0} C for 15 minutes. Lysates were then subject to centrifugation at 30,000g for 30 minutes. The supernatant was then aliquoted in 1 ml volumes and stored at – 80 ^{O}C.

To prepare GST fusion protein from crude extract, extracts were thawed on ice and transferred into 5 ml bijous. 1 ml of glutathione sepharose was added to each ml of extract and rotated at 4 ^{O}C for 3 hours. Glutathione beads absorbed to fusion protein were recovered by centrifugation, and the superntant was removed and stored. Fusion proteins were then eluted from the glutathione beads by rotation with 3x 1 ml of 20 mM mM glutathione for twenty minutes. Finally recovered protein was pooled and the concentration of the protein crudely determined via OD at 280nm and more accurately determined by Bradford assay (Biorad). Using 10 µl of protein serial dilutions in water were made and added to 50 µl Bradford reagent. Protein concentration was determined by optical density readings at 595 nm and comparison to a range of known protein standards (2 mg/ml – 0.05 mg ml). Finally purified proteins were run on an SDS PAGE gel to check the integrity, and molecular weight of the protein.

2.2.2 CELL CULTURE

JURKAT T CELL LYMPHOMA –J6

Jurkat J6 Lymphoma T cells were maintained in RPMI-1640, supplemented with 10% heat inactivated foetal calf serum, Penicillin (50U/ml), Streptomycin (50µg /ml), and fungizone. Cells were grown in suspension in 175cm^2 tissue culture flasks at 5% $C0_2$ in a humidified atmosphere at 37^{o}C in 175 cm^2 tissue culture flasks. Cells

were passaged 2-3 times weekly, by splitting each flask 1:4 into the original volume of pre-warmed full growth medium (as described above).

CTLA4$^+$ T CELL HYBRIDOMA

The murine T cell Hybridoma, DC27.1 which was stably transfecetd with CTLA4, was a kind gift from Chris Rudd (Dana Farber institute, USA), and was maintained in RPMI-1640 supplemented with 10% heat inactivated foetal calf serum, penicillin (50U/ml) streptomycin (50µg/ml), and Fungizone^{TM} (diluted 1:500). Cells were grown in 175cm^2 tissue culture flasks, which were incubated in a humidified atmosphere at 5% CO_2, 37°C. Cells grew in a semi-adherent manner, and adherent cells were removed from tissue culture plastic, prior to passageing, via vigorous agitation. The resulting suspension was split 1:4 into the original volume of fresh pre-warmed full growth medium as described above, 3 times weekly.

DC27.1 CD28$^+$ T CELL HYBRIDOMA

The murine T cell hybridoma DC27.1, stably expressing wild type or site mutated CD28 was kindly provided by Daniel Olive (INSERM, Marseille) and maintained in DMDM containing 10% FCS, supplemented with sodium pyruvate (1mM) and 2-mercaptoethanol (50µM) and penicillin (50U/ml) and streptomycin (50µg/ml) the cells were grown in a humidified atmosphere at 37^{0}C, 5% CO_2. Cells grew in a semi-adherent manner, and adherent cells were removed from tissue culture plastic, via to passageing, via vigorous agitation. The resultant suspension was split 1:4, three times weekly.

B7$^+$ CHO CELLS

CHO B7 stable transfectants and CHO parentals were a kind gift from Dave Sansom (University of Bath, UK), and were maintained in Dulbecco's modified essentials medium (DMEM), without glutamine, with pyridoxine (and supplemented with 10% heat inactivated foetal calf serum, nucleosides (GIBCO), and non essential amino acids (GIBCO). The cells were grown at 37°C, 5% CO_2. Cells were passaged at 100% confluency. After removing the medium from confluent cells the remaining medium was washed from the flask with PBS and cells were overlayed with trypsin-

EDTA, and placed at 37°C for 2 minutes. Trypsin-EDTA was rapidly neutralised with DMEM with 10% foetal calf serum, and cells were dislodged from plastic by vigorous agitation, and subsequently pelleted by centrifugation. Pelleted cells were resuspended in full growth medium, and 'split' 1:5 for subsequent culture.

A20 B CELL LYMPHOMA

The A20 B cell lymphoma was maintained in RPMI 1640, supplemented with 10% heat inactivated foetal calf serum, 50U/ml Penicillin, 50µg/ml Streptomycin, and 50µM 2-mercaptoethanol. The cells were grown at 5% CO_2, 37°C. Cells were grown until semi-confluent. Cells grew in a semi-adherent manner and aherent cells were harvested before passageing by vigorous agitation of the culture flask. The resultant suspension was split 1:3, three times a week.

ANALYSIS OF CELL SURFACE ANTIGENS

All cells were routinely analysed for expression of the relevant cell surface antigens via FACS analysis (See diagram 13). For each cell line to be analysed, $5x10^4$ cells were aliquoted and washed twice in serum free RPMI 1640, and resuspended in 100 µl of the appropiate antibody specific for cell surface antigens, at a dilution of 1 µg/ml in serum free RPMI 1640. Cells were subsequently incubated at 4 °C for 1 hour and agitated intermittently. As a control for non-specific binding, $5x10^4$ cells were incubated in 100 µl of an antibody which was isotype matched to the primary antibody, for 1 hour at a concentration of 1 µg/ml in serum free RPMI 1640.

Control and non-control samples were then washed twice using ice cold PBS, and incubated at 4° C for 20 minutes with 100 µl anti-Mouse-FITC, or anti Rabbit-FITC, depending on the species in which the primary antibody was raised, diluted to 250 µg/µl. Finally samples were washed three times in PBS and analysed for surface expression of antigenic determinants by flow cytometry.

INTRACELLULAR FACS STAINING

$CTLA4^+$ T cell hybridoma cells were analysed for extracellular CTLA4 expression (as described above) and intra-cellular expression of internalised CTLA4 protein.

$5x10^4$ cells were aliquoted and then washed twice in serum free RPMI 1640. Cells were subsequently fixed in 100 µl 1% paraformaldehyde for 15 minutes at room

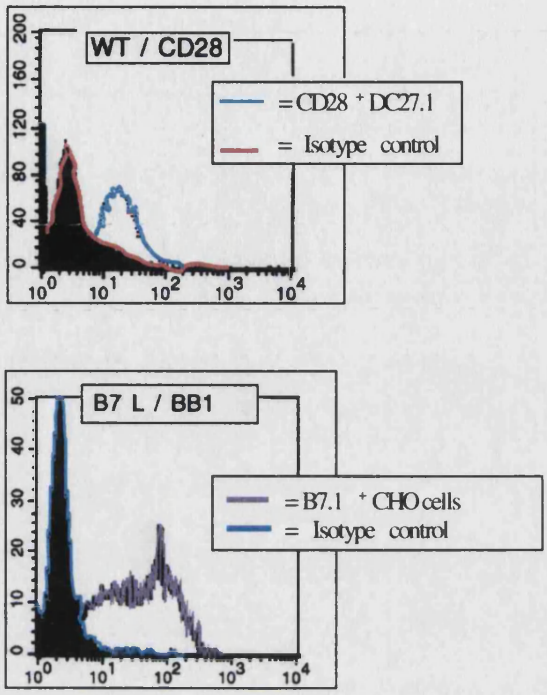


DIAGRAM 13 Analysis of cell surface antigens

temperature, before washing three times in PBS. The cells were then permeabilised in 100µl 0.01% saponin, for 15 minutes at room temperature. Subsequently the cells were washed three times in PBS and incubated for 1 hour with 100 µl anti-CTLA4 antibody, 11D4, diluted to a concentration of 1µg/ml in serum free RPMI 1640. As a negative control, cells were treated similarly and incubated with IgG2a, isotype matched immunoglobulins. Finally cells were washed three times in PBS and incubated for 20 minutes with anti-mouse FITC at a concentration of 250 µg/ml. Samples were then analysed for intracelllular CTLA4 expression, by flow cytometry.

FREEZING AND THAWING CELLS

Early passage cells of all cells lines were stored for long term use in liquid nitrogen. Prior to freezing cells were grown until semi-confluent and then pelleted by centrifugation and counted using a haemocytometer. Viability of cells was determined via trypan blue exclusion. Cells were resuspended in freezing mix: 10% dimethyl sulfoxide, 90% full growth medium and aliquoted at a cell density of

$5x10^6$/ml, into cryovials™. Subsequently cells were placed in 'MR Frosty'™ cooler at –80°C, which allowed the vials to cool at a steady rate of 1 °C per minute. After 12 hours cells were placed onto canes for storage in liquid nitrogen cylinders.

Cells were recovered from liquid nitrogen by removing from canes and placing on ice. Cells were then rapidly thawed in a 37 °C waterbath, and immediately diluted into pre-warmed full growth medium, and pelleted by centrifugation. The resultant pellet was resuspended in 10 mls of warmed full growth medium, and transferred into a 20 cm^2 flask, for overnight culture. Cells were subject to visual assesment over the subsequent days, and when confluent, and recovered, transferred in to 80 cm^2 and subsequently 175 cm^2 tissue culture flasks.

2.2.3 CELL STIMULATIONS

PREPARATION OF CELLS

Prior to stimulating, cells were grown to semi-confluency and harvested from tissue culture flasks. Where semi adherent cells were used, vigorous agitation was necessary to remove cells from tissue culture plastic. Where adherent CHO cells were used to stimulate T cell lines, cells were removed from tissue culture plastic using trypsin-EDTA, and incubated at 37 °C, for 2 minutes. Care was taken to neutralise the trypsin rapidly, so as to avoid cleavage of cell surface antigens.

All cells lines were then pelleted by centrifugation at 1200 r.p.m. at room temperature. Resultant cell pellets were pooled and washed four times in RPMI 1640, without serum. Finally, after having been assessed for viability via trypan blue exclusion and counted using a haemocytometer, cells were then resuspended at a density of $40x10^6$ cells per ml in HEPES buffered serum free RPMI 1640, and aliquoted in 0.5 ml volumes, into eppendorf tubes. Cells were then allowed to quiesce for 30 minutes at 37 °C, before being stimulated.

T CELL STIMULATIONS

T cell antibody stimulations (anti-CD28, anti-CTLA4, and anti CD3) were achieved using the appropriate antibody at a concentration of 5µg per 0.5 ml aliquot of cells.

Cells were immediately mixed and incubated at 37 °C for the time points required. T cells were stimulated via the CD28 physiological ligand in the following manner: CHO cells expressing B7.1, were prepared as described earlier and resuspended at a concentration of $24x10^6$ per ml, and then incubated at 37 °C prior to stimulation. To stimulate T cells, 0.5 mls of the $B7.1^+$ CHO cell suspension was added to 0.5 mls of T cells (prepared as above) such that the T cell: CHO cell ratio was 3:1. Cells were then mixed and briefly pulsed in a microcentrifuge, to achieve cell-cell contact, and then placed at 37 ° C for the required time points.

A20- B CELL STIMULATIONS

The A20 B cell Lymphoma was stimulated with either 40 µg/ml intact rabbit anti-mouse IgG (RAMIG) or 24 µg/ml F(ab')2 fragments of rabbit anti-mouse IgG(F(ab')2, for the time points required.

PREPARATION OF WHOLE CELL EXTRACTS

Following stimulation, cells were removed from the waterbath, and pulsed for 5 seconds in a microcentrifuge, to sediment. The medium was then aspirated from the cells, and the cells were then lysed in 500 µl 1x cell lysis buffer [0.5% v/v NP40, 65mM NaCl, 10mM Tris pH7.5, 1%β glycerophosphate, 5 mM iodoacetamide, 5 mM NaF, 1mM phenylmethyl-sulfonylflouride, 1 µg/ml leupeptin, 1 µg/ml pepstatinA, 1µM sodium orthovanadate]. All samples were incubated on ice, to allow thorough lysis to occur, after which nuclear debris was sedimented via centrifugation at 4 °C, 13,000 r.p.m, for 15 minutes. Subsequently, the supernatants, containing cellular proteins, were harvested from the samples, and kept on ice.

MEMBRANE CYTOSOL FRACTIONATION

For membrane cytosol fractionation cells were stimulated and lysed in lysis bufer minus NP40. Cells were then sonicated ten times for 15 seconds on ice and intact cell debris was pelleted by centrifugation for 30 seconds at 13,000rpm in a bench top microcentrifuge. Supernatants were harvested and membrane cytosol fractions prepared by centrifugation at 300,000g (100,000 r.p.m. using the Beckman T 120 ultracentrifuge rotor), for 20 minutes at 4 °C. Supernatants containing cytosolic

fractions were harvested and boiled in Laemmli sample buffer, or subject to immunoprecipitation as described below. Pellets, containing membrane fraction, were washed using detergent free lysis buffer and then solubilised in lysis bufer plus 10% NP40. Membrane fractions were then either boiled in Laemmli sample buffer or subject to immunoprecipitation as described below.

IMMUNOPRECIPITATION FROM CELL LYSATES

Where immunoprecipitation was to be carried out, samples were first pre-cleared by the addition of 20 µl 50 % protein A sepharose slurry and rotated for 20 minutes at 4 °C. After pre-clearing, protein A sepharose was sedimented by centrifugation at 13,000 r.p.m. for 1 minute, and the supernatants were harvested. Cell lysates were next subject to immunoprecipitation by addition of the relevant antibody at a final concentration of 2 ng/ml, and rotation for 2 hours at 4 ° C. Immune complexes were precipitated by rotation with 40 µl 50 % protein A sepharose slurry for 1 hour at 4 °C. Sepharose beads were then sedimented from cell lysates, by pulsing tubes to 13,000 r.p.m in a microcentrifuge. Immunoprecipitates were then washed 5 times with lysis buffer, drained with a hamilton syringe, and boiled in 30 µl SDS-Laemmli sample buffer for 10 minutes.

ACETONE PRECIPITATION OF PROTEINS

Acetone precipitation of total cell proteins was achieved, following sedimentation of nuclear proteins from the lysed cells, through the addition of 0.7 volumes of ice cold acetone to the cell lysate. Samples were placed at –20 °C for one hour and then flocculent protein pellets were sedimented via centrifugation at 13, 000 r.p.m, for 20 minutes. Pellets were retained and dried *in vacuo*. Finally acetone precipitated proteins were boiled in 200 µl SDS-Laemmli sample buffer, and loaded at a volume of 10 µl per gel.

GST-FUSION PROTEIN PRECIPITATES

Where precipitation using a GST fusion protein was required, 20 µg of GST fusion protein was added to cell lysates and samples were rotated for 4 hours at 4 ° C.

Subsequently, protein complexes were adsorbed through the addition of 30 µl GST sepharose slurry, and rotation for 1 hour at 4 °C. Adsorbed proteins were recovered via sedimentation of the sepharose beads by centrifugation at 13.000 r.p.m., followed by 5 washes with lysis buffer. Finally beads were sedemented and drained with a hamilton syringe, prior to boiling in 30 µl SDS-Laemmli sample buffer.

2.2.4 WESTERN BLOTTING

SDS- PAGE

Immunoprecipitated proteins, fusion protein precipitates, and acetone precipitates, were separated via 7-17 % gradient SDS PAGE overnight, or 7.5 % SDS-PAGE for one hour. All gels were assembled using 1.5 cm spacers.

Proteins were then transferred onto nitro cellulose membranes by semi dry transfer. Eight pieces of 3 MM filter paper were cut to the exact size of the gel, and each piece was wetted with in semi dry transfer buffer and placed onto the anode of the semi dry transfer apparatus. Between sheets each filter paper was vigorously rolled to remove air bubbles. Finally after four sheets had been layered onto the anode in a vertical stack, nitrocellulose which had been cut to the exact size of the gel and pre-soaked in semi-dry transfer buffer, was layered onto the stack, and rollered. The gel was briefly pre-soaked in semi dry transfer buffer, placed onto the nitro-cellulose membrane and gently rollered. A further four pieces of pre-wetted 3 MM filter paper were layered and rollered. Finally the semi-dry transfer cathode was applied and proteins were set to transfer for one hour and a half at 1 mA per cm^2.

IMMUNOBLOTTING

Following transfer blots were rinsed in dH_2O, and proteins were visualised using ponceau-S stain, and were subsequently de-stained with dH_2O. Nitro-cellulose membranes were blocked for 3 hrs with 5 % Marvel in PBS. Primary antibodies were diluted in 0.05% Marvel (in PBS) at a final antibody concentration of 1ng/ml. Primary antibodies were applied for three hours at room temperature in the case of anti-phospho tyrosine antibody 4G10, or overnight in the case of all other antibodies.

After removal of the primary antibody, and its storage at 4 °C for re-use, blots were washed 3 times for 15 minutes each wash, in PBS.

After washing antibody bound proteins were detected using a secondary antibody consisting of horseradish peroxidase-conjugated immunoglobulins, relevant to the species in which the primary antibody had been raised. Secondary antibody stocks were diluted at 1:20,000 in 0.05% marvel, and applied for 40 minutes. Following removal, secondary antibodies were discarded and blots were washed thoroughtly six times for 15 minutes each wash, in PBS. Blots were visualised using chemi-luminescence reagent (ECL), and autoradiography.

FAR WESTERN BLOTTING

GST fusion proteins were used to far western blot nitro cellulose membranes which had been prepared as described above. GST Fusion protein was diluted in 0.05 % marvel at a concentration of 100 ng per ml, and incubated at room temperature with the blocked membrane, for three hours. Subsequently blots were washed five times with PBS / 0.01 % Tween, for five minutes each wash. Membranes were then incubated at room temperature with anti-GST antibody, at a dilution of 1: 10,000, for one hour. Blots were then washed three times with PBS / 0.01 % Tween and incubated for one hour at room temperature with anti- mouse HRP conjugated immunoglobulins, diluted to 1:5000 in 0.05 % marvel. Finally blots were washed three times for five minute in PBS / 0.01 % Tween and visualised with ECL by autoradiogram.

STRIPPING AND REPROBING

Prior to reprobing, or storage, membranes were stripped at 50 °C for 1 hour in stripping buffer with fresh 2- mercaptoethanol. After stripping blots were washed 10 times, in PBS, each wash for 15 minutes. Where blots were to be stored they were air-dried between two sheets of 3 MM paper, and stored at room temperature. Where blots were to be re-probed they were blocked in 5% Marvel for 1 hour.

2.2.5 IN VITRO LIPID KINASE ASSAY

Immunoprecipitated proteins from stimulated whole cell extracts were prepared as described above. Following washing in lysis buffer, protein A sepharose beads were subject to further washing: once in ice cold PBS, twice in 0.5 mM LiCl, 100 mM Tris-HCL(pH 7.6), once in dH_2O and finally once in lipid kinase buffer [5 mM $MgCl_2$, 0.25 mM EDTA, 20 mM HEPES pH 7.4]. Ptd ins activity was determined via the method described by Whitman et al (1988). Following washing the sepharose beads were resuspended in 30 mls of lipid kinase buffer, and 50 µl of a lipid mixture, sonicated in 25mM HEPES buffer pH7.4 and 1mM EDTA, containing 0.1mg/ml phosphatidyl- inositol and 0.1 mg/ml phosphatidyl serine,) was added.

The lipid kinase reaction was initiated by the addition of 20 µCi of [γ-^{32}P] ATP and 100µM ATP and terminated after 15 mins. by the addition of 80 µls 1M HCl and 200µl chloroform:methanol in a 1:1 ratio. After vigorous mixing and centrifugation to separate the phases, the organic layer was removed, dried *in vacuo* and resuspended in 50 µl chloroform. The extracted lipids were then analysed by thin layer chromatography in propan-1-ol:acetic acid (2N) 65:35 v/v developing solvents, and visualised by exposure to iodine vapour and autoradiography.

2.2.6 IN VITRO PROTEIN KINASE ASSAYS

Analysis of protein kinase activity in immunoprecipitated protein complexes prepared from stimulated whole cell extracts, as described above, was carried out via an *in vitro* protein kinase assay. After washing sepharose beads in lysis buffer following immunoprecipitation, they were further washed in protein kinase assay buffer lysis buffer (100 mM NaCl, 25 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 5 mM $MnCl_2$. 100 µM sodium orthovanadate). *In vitro* kinase activity was initiated by the addition of 20 µl kinase assay buffer containing 10 µM ATP and 10 µCi of [γ-^{32}P]-ATP. Reactions were quenched after 10 minutes by the addition of 1 ml of lysis buffer containing 20 mM EDTA. The immuno-precipitates were then washed 8 times in this buffer, to remove unincorporated radioisotope and then sepharose beads were drained. Samples were boiled in SDS-Laemmli sample buffer and separated via 7-17

% SDS-PAGE, overnight. Gels were fixed in propan-2-ol:H_2O:acetic acid (50:130:20) for 20 minutes then briefly rinsed in water and dried down at 80 ^{o}C under Vacuum, for 1 hr. Radiolabelled kinase assay products were visualised by autoradiography at –70 °C.

2.2.7 IMMUNOFLUORESCENCE STAINING.

To visualise cellular proteins via confocal microscopy, cells were plated at $1x10^6$ cells per ml onto poly-L Lysine coated cover slips placed in 24 well plates and left to adhere overnight under normal growth conditions. Subsequently cells were stimulated as described above, and signalling was quenched via fixation in 1 % paraformaldehyde, for 15 minutes at room temperature. Samples were then permeabilised in ice cold acetone for 5 minutes and then rinsed with PBS / 0.01 % Tween prior to blocking in abdil solution [PBS / 0.01 % BSA / 1 % foetal calf serum], for 10 minutes at room temperature. Samples were then washed 3 times with PBS / 0.01 % Tween, for five minutes each wash. After the final wash, primary antibodies were applied, at a concentration of 1:1000, diluted in Abdil buffer, and samples were incubated for 30 minutes at room temperature, using a humidified slide chamber, to prevent evaporation.

Subsequently samples were washed 3 times in PBS / 0.01 % Tween, for 5 minutes each wash, and incubated with secondary antibody, FITC conjugated anti- rabbit immunoglobulins, for 20 minutes at room temperature, in the dark. Samples were then washed a further 3 times with PBS / 0.01 % Tween and then mounted with 50 % glycerol in PBS containing 2 % Dabco, and dried for one hour. Samples were visualised via immunoflourescence microscopy using a 'Zeiss Axiovert' 100 M inverted epiflourescence microcope attached to a LSM 510 confocal laser scanning system equipped with a krypton / argon laser (Karl Zeiss, Oberkochen, Germany).

2.2.8 CELL TRANSFECTIONS

Prior to electroporation, Jurkat T cells were grown to semi confluency and then harvested by centrifugation at 1200 r.p.m. Cell pellets were pooled and counted using a haemocytometer, assessing viability via Trypan Blue exclusion. Cells were

then aliquoted in 500 µl volumes at 15 x 10^6 cells per ml, into electroporation cuvettes, and electroporated with 30 µg DNA per cuvette, at 960 µF, 310 mV using a Biorad 'gene pulser' electroporation system. In cases where green flourescent protein expressing plasmids were used, 10 µg DNA was added per cuvette. Following electroporation, cells were placed directly into culture at 50 x 10^6 cells per 10 mls full growth medium supplemented with 10 % fetal calf serum, and pre-equilibrated to 37 °C, 5 % FCS.

2.2.9 CONFOCAL VISUALISATION OF GFP TAGGED PROTEINS.

To assess the re-distribution of GFP-PH PKB through co-expression with rCD2 SHIP, and rCD2 C^{671}/A SHIP throughout the cell, Jurkat cells were electroporated as described above, and plated onto poly-L Lysine coated coverslips in 24 well plates. After 5 hours in culture cells adhered to cover slips were fixed in 1 % paraformaldehyde, and mounted with 50 % glycerol in PBS containing 2 % Dabco, prior to visualisation using a Zeiss Axiovert 100M confocal microscope.

2.2.10 INOSITOL (POLY)PHOSPHATE 5-PHOSPHATASE ASSAY

To assess the inositol polyphosphate 5-phosphatase activity of endogenous SHIP derived from human CD28- or murine CD3- stimulated DC27.1 cells, cells were prepared and stimulated as described above prior to immunoprecipitation with anti-SHIP polyclonal antiserum. To assess the inositol polyphosphate 5-phosphatase activity of CD2-SHIP and CD2-C^{671}/A SHIP, cells were electroporated as described above and immunoprecipitated with anti CD2 antibody, OX34.

Immunoprecipitates were assayed for 5-phosphatase activity by determining. the *in vitro* hydrolysis {[^{3}H]- Ins(1,3,4,5)P_4} to [^{3}H]- inositol [1,3,4]- trisphosphate {[^{3}H]- Ins(1,3,4,)P_3}. Immunoprecipitates were re-suspended in 25µl containing 16µM [^{3}H]- Ins(1,3,4,5)P_4 under conditions where the reaction was linear with time (20 mins, 37^{0}C). Reactions were stopped by the addition of acidified: chloroform: methanol and the aqueous phase was harvested and dried under vacuum. Finally the samples were resuspended in 100 µl of water. The samples were then analysed by anion-exchange HPLC analysis using a Partisphere SAX column (Whatman) and

levels of [^{3}H]- Ins(1,3,4,5)P_4 and [^{3}H]- Ins(1,3,4,)P_3 were quantitated using an on-line radiodetector (Canberra-Packard].

2.2.11 MEASUREMENT OF D3 PHOSPHOINOSITIDE LIPIDS.

To measure D3 inositol lipid accumulation on intact cells, cells were first harvested from culture and depleted of phosphate by washing three times in 50 mls of phosphate free DMEM, and incubating at 37°C for 15 minutes between washes. After phosphate depletion, cells were resuspended at $20x10^6$ cells per ml in RPMI 1640 supplemented with 5% dialysed FCS and 20 mM HEPES prior to labelling with 1 m Ci [^{32}P]- phosphoric acid, at 37^{O}C for four hours.

Following incubation cells were washed three times in phosphate free DMEM, to remove unincorporated [^{32}P]-phosphoric acid, and then re suspended at $20x10^6$ cells per ml in DMEM and aliquoted into 500µl volumes. Cells were then stimulated in as described above. Reactions were quenched with ice cold Methanol:H_2O:chloroform, producing a homogenous primary extraction phase.

Phases were then separated by the addition of 200 µl folsch lpids in trace amounts, suspended in chloroform, and 200 µl 5 mM TBAS / 2.4 M HCl. Tubes were then vortexed and phases were separated by centrifugation at 800 r.p.m for 5'. Lower phases were harvested into a fresh tube using a gel loading tip, and 400 µl of 0.1 M HCl, 5 mM EDTA was added. The samples were then vortexed and centrifuged as before. Again the lower phase was harvested into a fresh tube and dried *in vacuo*. Once dried the extracted lipids were deacylated by the addition of 1 ml 25 % w/v methylamine / methanol / butan-1-ol (4:4:1) followed by incubation at 53 °C for 40 mins. Samples were then cooled on ice for 5 mins and dried *in vacuo*. Samples were then resuspended in 0.5 ml H_2O followed by addition of 0.6 ml Butan-1-ol / petroleum ether (bp 40-60 °C) / ethyl formate (20 / 4 / 1 v/v) to the dried deacylated lipids. Samples were then vortexed and centrifuged 800 r.p.m. before removing the as upper organic phase and washing the lower, water soluble phase with a further 0.6 ml of Butan-1-ol / petroleum ether (bp 40-60 °C) / ethyl formate mix. After vortexing, centrifugation as before the upper phase was discarded and the lower

phase was dried *in vacuo*. Finally pellets were redissolved in 100 µl dH_2O, and analysed using a 12.5 cm Whatman Partisphere SAX column.

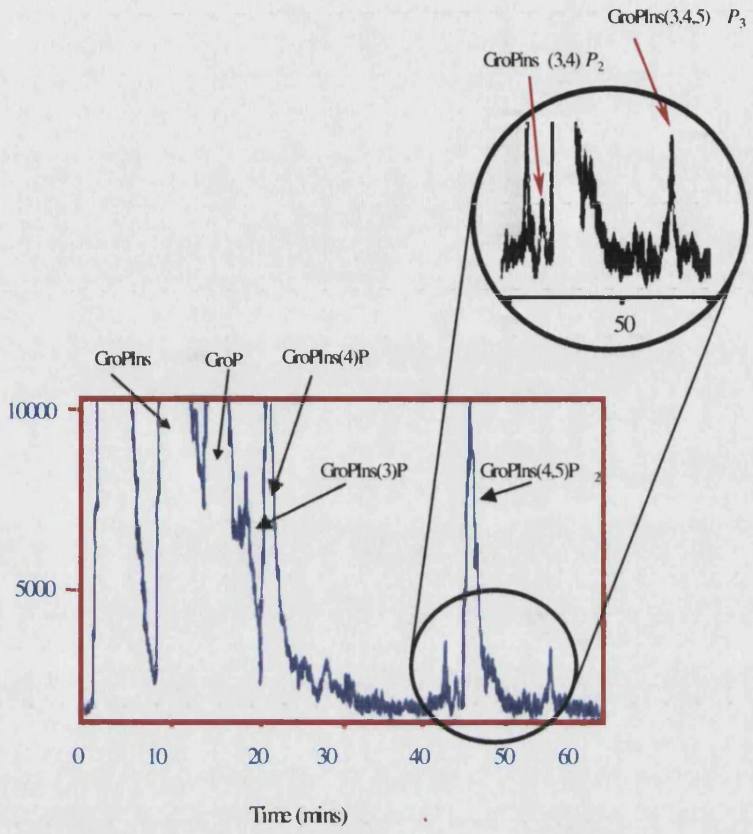


Diagram 14: Sample HPLC elution trace of deacylated [^{32}P]-labelled phosphoinostide lipid products derived from resting Jurkat T cells.

Samples were eluted from the column using a gradient based on buffers A (H_2O) and B (1.25 M (NH_4) HPO_4] (adjusted to pH 3.8 with H_3PO_4 at 25 °C) at a flow rate of 1 ml / min: 0min, 0 % B; 5 min, 0 % B; 45 min, 12 %B; 52 min, 20% B; 64 min, 100% B; 70 min, 100% B; 71 min, 0 % B 90 mins, 0 % A and B.

2.2.12 LYMPHOCYTE PREPARATION AND PURIFICATION

Blood from healthy donors was taken aseptically in 50 ml syringes containing heparin at 10 U per ml of blood, via 19 gauge butterfly needles. The blood was diluted immediately 1:1 in RPMI 1640 culture medium and 35 ml aliquots were carefully layered onto 15 ml lymphoprep, so as not to disturb the interphase, in 50 ml centrifuge tubes and spun without brake for 30 minutes at 1200 rpm.

Lymphocytes were then carefully removed from the interphase and washed x3 in RPMI. To prepare cultured lymphoblasts, cells were then resuspended in an equal volume to the blood from which they originated and incubated at 37 °C, 95 % humidity and 5 % CO_2 for 72 hours in 80 cm_2 tissue culture flasks with one of the following mitogenic stimuli: 1 µg/ml Staphylococcal enterotoxin B, 1 µg/ml phytohaemagglutinin A (PHA), 1 µg / ml Ionomycin with 5 ng / ml phorbol myristate acetate or CD2/CD28 ligation. After 72h and every 48 h for 15 days the blasts were supplemented with 20 ng / ml Il-2. T blasts were maintained at a 0.5-1.5x 10^6 per ml density. To prepare purified T-lymphocytes the PBMCs were resuspended in volumes equal to the volume of blood from which they originated in complete RPMI 1640, 10 % FCS, pen.strep. (500 U / ml) and 0.5 µg / ml Amphotericin B. Adherent cells were removed by plastic adherence on tissue culture petri dishes or 175 cm^2 flasks incubated at 37 °C for 60 mins, 95 % humidity and 5 % CO_2 .

The non adherent cells were washed gently from the surface of the plate and resuspended in 15 ml centrifuge tubes in RPMI (1ml for PBMCs from 100ml of blood), and rotated gently for 45' at 4°C with the following mouse α human Abs at 1µg/ml: anti B Cell mAb against CD19, anti monocyte and macrophage Ab clone UCHM-1 against CD14, and anti APC Ab L243 against MHC D-R. The tagged cells were then washed and resuspended with 2-4 x10^6 /ml magnetic beads M-450, coated with sheep anti mouse IgG Abs (in 2mls for 100mls of blood). After 45' rotation at 4°C the cells were purified over magnetic beads in 12ml RPMI. Cells were counted and viability assessed with trypan blue exclusion.

2.2.13 PROLIFERATION ASSAY

Purified T cells were resuspended in RPMI growth medium, and aliquoted, 150µl/5x10^4cells/well in 96 well plates, and stimulated with varying concentrations of αCD3, αCD3, αCD28 or CHO-B7.1, PMA ionomycin or PHA and IL-2 the various points were plated in quintiplicate to standardise the assay. Antibodies were used soluble with cross linker at 1-10 µg/ml. All other agents were used at the concentration stated previously. Inhibitors were used at 0.1 -100 nM concentrations.

The plates were incubated at 37°C, 95% humidity and 5% CO_2 for 48 h and then pulsed with 0.5μCi/well ^{3}H-thymidine. 72h after stimulation the cells were harvested using an automated cell harvester on 96 well filter plates (Unifilter™) and radioactivity was measured with a Packard instruments β scintillation counter (Top Count™) according to manufacturer's specifications.

2.2.14 MEASUREMENT OF INTRACELLULAR [Ca^{2+}]

Using the calcium-sensitive fluorochrome Fura-2 AM intracellular [Ca2+] can be readily measured. Fura-2 is coupled to the acetoxymethylester (AM) which allows entry into cells, and is then cleaved by endogenous esterases, preventing the escape of fura-2 from the cells. On binding to calcium ions, the fluorescence excitation maximum of fura-2 transfers to a lower wavelength without any alteration in the emission spectrum, thereby enabling fura-2 to be used as a dual excitation indicator. The excitation maximum for free-calcium and bound-calcium can be measured at 380nm and 340nm respectively.

CALIBRATION OF THE FLUORIMETER

Calibration was obtained by monitoring the fluorescent changes in the cell suspensions after the addition of 0.16μg/ml digitonin (to lyse cell membranes and expel intracellular calcium). Fluorescence was monitored using a dual excitation/dual emission spectrofluorimeter (Photon Technologies) for 30 seconds followed by the addition of 40mM sodium hydroxide (NaOH) and 4mM EGTA. Conversion of the fluorescence ratios at two wavelengths into intracellular calcium concentration was achieved using the equation developed by Grynkiewicz *et al* (Grynkiewicz *et al*, 1985):

$$[Ca2+]i = Kd \frac{R-R_{min}}{R_{max}-R} \frac{S_{f2}}{S_{b2}}$$

Where,

- Kd = effective dissociation constant for fura-2 (2.24×10^{-7} M)
- R = Ca^{2+} bound/Ca^{2+} free fluorescence ratio
- Rmin = fluorescence ratio with zero calcium
- Rmax = fluorescence ratio under saturating conditions

- S_{12}/S_{b2} = ratio of fluorescence values for Ca^{2+}/Ca^{2+} free indicator measured at the wavelength used to monitor Ca^{2+} - free indicator (denominator wavelength of R) i.e. at 380nm.

LOADING CELLS WITH FURA-2/AM

Cells were pelleted by centrifugation and resuspended in HBSS (without calcium, magnesium and phenol) containing 0.1% BSA at $1x10^7$ cells per ml. Cells were then incubated with 5μM fura-2/AM at 37°C for 45 minutes. Following incubation, cells were washed twice and resuspended at $1x10^6$ cells per ml in HBSS/BSA.

$[CA^{2+}]_I$ MEASUREMENT

Fura-loaded cells were aliquoted into a 2-ml cuvette at a concentration of $1x10^6$ - $2x10^6$ cells per ml. To the cell suspension 1mM of calcium chloride and 1mM of magnesium chloride were added and allowed to equilibrate at 37°C in the spectrofluorimeter for 5 minutes.

Prior to the addition of the agonists, a basal calcium measurement was taken for 30 seconds. The agonist induce-response was monitored for at least 180 seconds and was detected using dual excitation wavelengths of 340nm and 380nm and a single emission wavelength of 510nm on the spectrofluorimeter (Photon Technologies).

3. RESULTS –T LYMPHOCYTES

3.1 CD28 AND PI3K

As outlined by the introduction to this study previous studies have examined the role of CD28 in activating PI3K dependent signalling cascades in T cells (Ward et al 1996). This study has further examined the regulatory mechanisms by which these pathways are governed. Firstly, the potential regulatory role of the multiple catalytic isoforms of PI3K, p110α, p110β, and the leukocyte expressed p110δ, in the context of CD28 signalling was considered. Using the acute lymphoblastic T cell line, Jurkat, the association of these isoforms with CD28, in response to B7 ligation, is described.

DEMONSTRATION OF THE RAPID ACCUMULATION OF PI(3,4,5)P_3 IN RESPONSE TO B7.1 LIGATION OF CD28.

For the purposes of this study the Jurkat T cell line, J6, was used to investigate CD28 driven PI3K dependent signalling cascades. Ligation of CD28, by the physiological ligand B7.1 was achieved here using a B7.1 expressing CHO cell line, (CHO-B7.1$^+$). Ligation of CD28 by its physiological ligand B7.1 has been previously described (Ward et al 1996) to lead to the rapid and sustained accumulation of the PI3K product, PI(3,4,5)P_3. Thus, to verify that B7.1 stimulated the biochemical coupling of the CD28 receptor to PI3K activation, the accumulation of D3-phosphoinositide lipids in response to stimulation of Jurkat T cells by CHO-B7.1$^+$ cells, was measured.

Firstly, it was noted that considerable levels of PI(3,4,5)P_3 could be measured in resting Jurkat T cells. Secondly, and in accordance with prior studies (Ward et al 1996), the rapid accumulation of PI(3,4,5)P_3 was observed in response to B7.1 stimulation of Jurkat T cells, which rose by a seven fold increase, above non-stimulated levels, after ten minutes following CD28 ligation (figure 1).

DETECTION OF PI3K REGULATORY AND CATALYTIC SUB-UNITS IN JURKAT T CELLS.

The different catalytic isoforms of Class 1A PI3Ks, p110α, β, and δ were described earlier, in the introduction to this study. In order to investigate the role of these isoforms in PI3K regulated signalling pathways in T lymphocytes, the expression of these isoforms in the Jurkat T cell line was first confirmed.

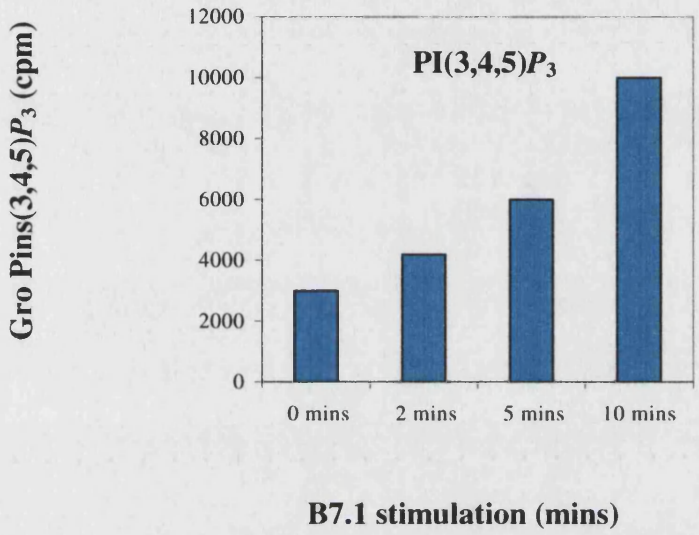


Figure 1: Accumulation of D3 Glycero-phosphoinositides in response to B7 ligation.

$2x10^7$ Jurkat T cells were labelled per point with [^{32}P]Pi in phosphate free medium supplemented with 10% dialysed FCS and HEPES, for 3 hrs at 37°C. Cells were then stimulated with B7.1 expressing CHO cells at a ratio of 3 Jurkats:1 CHO cell, for the times shown. Reactions were quenched with chloroform:methanol, and the phosohatidylinositol lipids were extracted, de-acylated and the [^{32}P]-labelled glycerophosphoryl derivatives of D-3 phosphoinositide lipids were analysed via anion exchange HPLC. Results are plotted as actual peak area. **Actual counts** = B7.1 0 mins: 3,100 cpm; B7.1 1min: 4,109cpm; B7.1 5min:6,087cpm; B7.1 10 minutes: 10,047cpm. Total counts 2,156,000cpm.
These data are from single experiment which is representative of three others.

The regulatory PI3K subunit, p85, and the catalytic isoforms p110α, p110β and p110δ were each readily detected in acetone precipitated (A.P) whole cell extracts (WCE) derived from Jurkat T cells (figure 2). Furthermore the ability of these catalytic isoforms to co-precipitate with p85 in non-stimulated T cells was examined. Coprecipitation of p110β and p110δ in p85 immunoprecipitates was observed (figure 2), whilst the p110α isoform appeared to co-precipitate less readily, and this isoform was observed only once to co-associate with p85 in five repetitions of this experiment.

DETECTION OF p110δ EXPRESSION IN CULTURED T LYMPHOBLASTS AND FRESHLY ISOLATED T LYMPHOCYTES.

Other groups (Chantry et al 1997, Vanhaesebroeck et al 1997) have previously reported the leukocyte restricted tissue distribution of the p110δ catalytic isoform of PI3K. Thus, in addition to the Jurkat cell line, expression of this isoform in primary isolated and cultured T cells, would be expected. To confirm whether this was the case, the presence of p110δ protein in WCEs and A.Ps. derived from human T lymphoblasts, which had been cultured for 7 days with IL-2, and from freshly isolated T lymphocytes purified from peripheral blood mononuclear cells (PBMCs) was examined (figure 3).

p110δ protein was readily detectable in WCEs and APs derived from both freshly purified T cells and cultured T lymphoblasts. Furthermore a p85α-p110δ recombinant protein was detected by anti-p110δ immunoblotting, whilst p85α-p110α was not (proteins kindly supplied by B.Vanhaesebroeck, Ludwig institute, London), thus confirming the specificity of the anti-p110δ antibody. (N.B. High background signal surrounding the p85α/p110α lane, due to overexposure of blots, slightly impairs visualisation of this result (figure 3)).

COASSOCIATION OF p110 ISOFORMS WITH CD28.

The direct co-association of the Class 1A PI3K regulatory subunit, p85 with conserved tyrosine phosphorylated motifs, present within the intracytoplasmic tail of CD28, has been described in previous studies by this group (Ward et al 1996) to occur in response to B7.1ligation of CD28. Following the detection, by this study, of p110β and p110δ and to a lesser extent p110α, catalytic isoforms of PI3K in various T cells, it was speculated that p85 may convey the differential recruitment of p110 isoforms to the CD28 co-receptor in response to ligation by B7.1. To verify whether this might be the

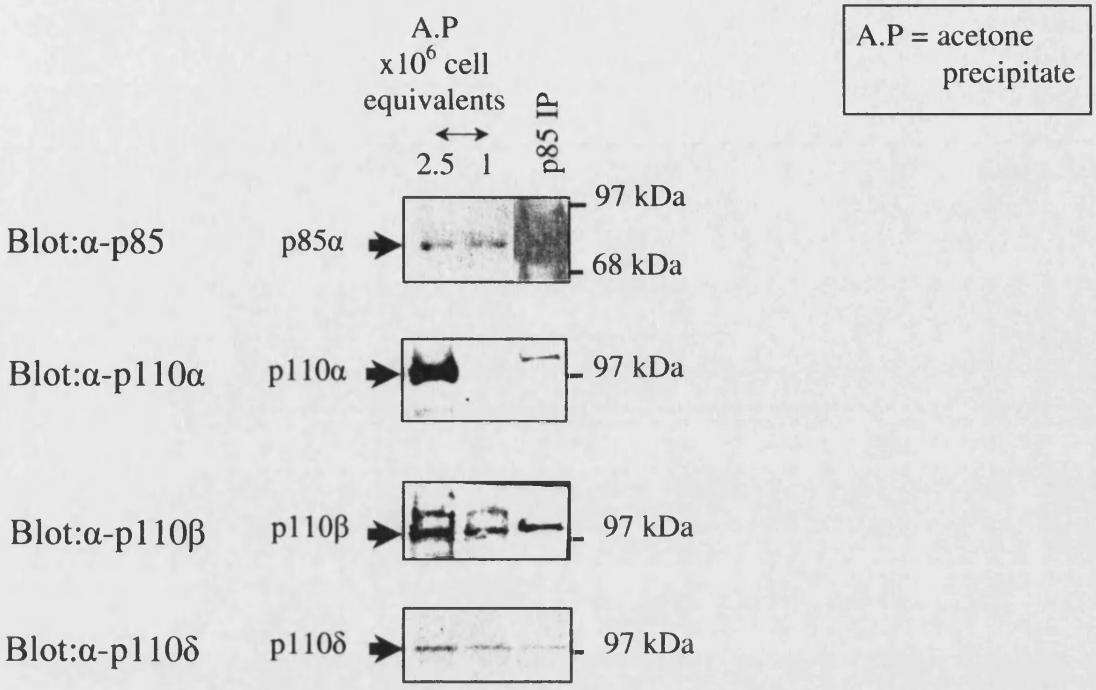


Figure 2: Presence of p85α and p110 isoforms in Jurkat T cell lysates.

Jurkat T cells were aliquoted at the cell numbers indicated and lysed prior to acetone precipitation on ice for one hour. Proteins were then pelleted by centrifugation at 13,000 rpm, 4°C, for 15 minutes. Resultant protein pellets were boiled in Laemmli-SDS sample buffer (A.P.s), and separated by 7.5% SDS-PAGE. In addition $2x10^7$ cells were lysed and immunoprecipitated with 1 μg anti-p85α antibody as a control, and the resultant precipitates were electrophoresed in parallel with the A.P.s. Separated proteins were transferred onto nitro-cellulose for immunoblotting with anti-p85α (top panel) or anti-p110 α (second panel), β (third panel), and δ (bottom panel) antibodies, each at a concentration of 1 μg per ml in 0.05% marvel/PBS, and proteins were visualised via chemiluminescence. These data are from a single experiment which is representative of five other experiments.

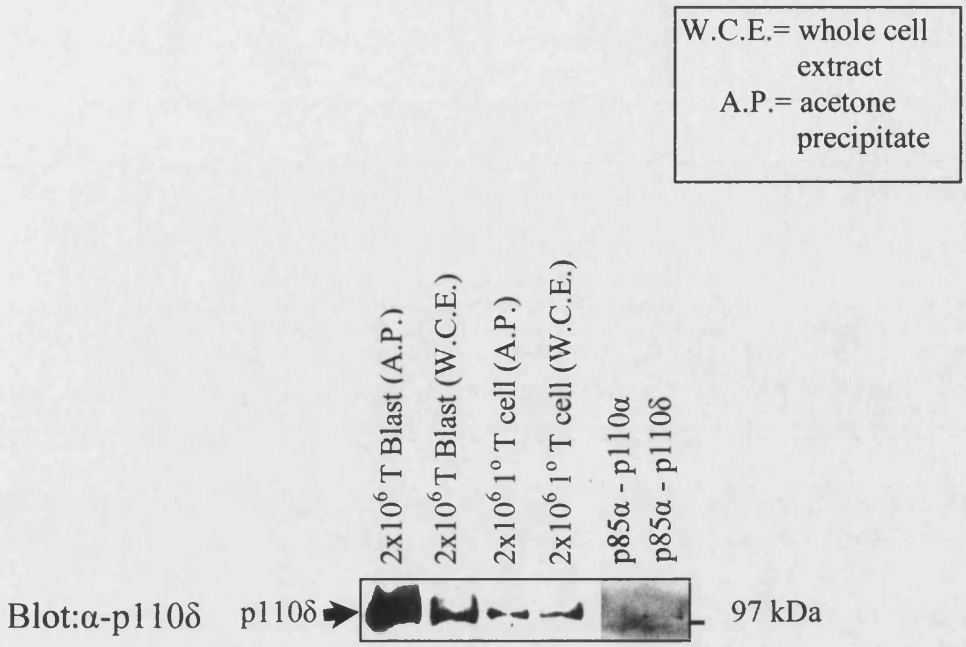


Figure 3: Presence of p110δ in purified T Cells, and T Lymphoblasts.

$2x10^6$ cultured T lymphoblasts (T blasts) or freshly isolated purified T cells (1° T cell) were lysed and whole cell extracts (WCE) were either boiled in Laemlli- SDS sample buffer, or precipitated on ice for one hour with 1.2 volumes of acetone, pelleted by centrifugation at 13,000rpm for 15 minutes (A.P), before boiling in Laemmli SDS-sample buffer. 0.01μg p85α-p110α and p85-p110δ recombinant proteins were electrophoresed in parallel as a control. Samples were separated by 7.5% SDS-PAGE. Proteins were then transferred to nitro-cellulose for immuno-blotting with anti p110δ mAb at a concentration of 1 μg per ml in 0.05% marvel/PBS, and proteins were visualised using chemiluminescence. These data are from a single experiment and are representative of three other experiments.

case, Jurkat T cells were stimulated with CHO-B7.1$^+$ cells, and CD28 was immunoprecipitated with the mAb 9.3. Immunoprecipitates were then subjected to immunoblot analysis with mAbs specific for each PI3K catalytic isoform.

Anti-p110δ immunoblot analysis identified the co-precipitation of p110δ protein with anti-CD28 immunoprecipitated proteins at 1 minute following B7.1 ligation (bottom panel, figure 4), and this co-precipitation was further enhanced at 5 minutes. Meanwhile anti-p110β immunoblotting failed to detect co-precipitation of p110β protein with anti-CD28 immunoprecipitates until 5 minutes following ligation. Over several experiments, the identification of p110α protein in anti-CD28 immuno-precipitates was either minimal (as shown in figure 4) or undetected, however recognition of a p85α/p110α recombinant peptide confirmed the efficacy of the anti-p110α antibody (*second panel*, figure 4). Finally the co-precipitation of p85 with anti-CD28 immunoprecipitates at 1 minute following ligation by B7.1, which was detected by immunoblotting with anti-p85α antibody, and was sustained over five minutes, demonstrated the efficient coupling of PI3K with CD28 in response to CHO-B7.1$^+$ stimulation of Jurkat T cells.

DETECTION OF PHOSPHO SERINE P110δ IN CD28 IMMUNO-PRECIPITATES.

The p110δ isoform of PI3K is known to have intrinsic protein kinase activity which serine phosphorylates the p110δ catalytic domain at Ser ^{1039} resulting in the down regulation of its lipid kinase activity (Vanhaesebroeck et al 1997). To examine the effects of CD28 ligation by B7.1 on p110δ serine phosphorylation, mAbs specific for phospho-p110δ (anti-p110δP) were used to immunoblot anti-CD28 immunoprecipitates derived from Jurkat T cells. In this way the comparative association of p110δ and phospho -p110δ with CD28 was ascertained.

Immunoblot analysis using anti-phospho-p110δ antibody detected the co-precipitation of phospho-p110δ with anti-CD28 immunoprecipitates from Jurkat T cells, which was maximal at 5-10 minutes following with CHO-B7.1$^+$ cells (*bottom panel*, figure 5), and had partially diminished after 15 minutes stimulation (*bottom panel*, figure 5). Immunoblotting of anti-CD28 immunoprecipitates, derived from CHO-B7.1$^+$ stimulated Jurkats, using anti-p110δ Ab revealed the co-precipitation of p110δ protein with CD28

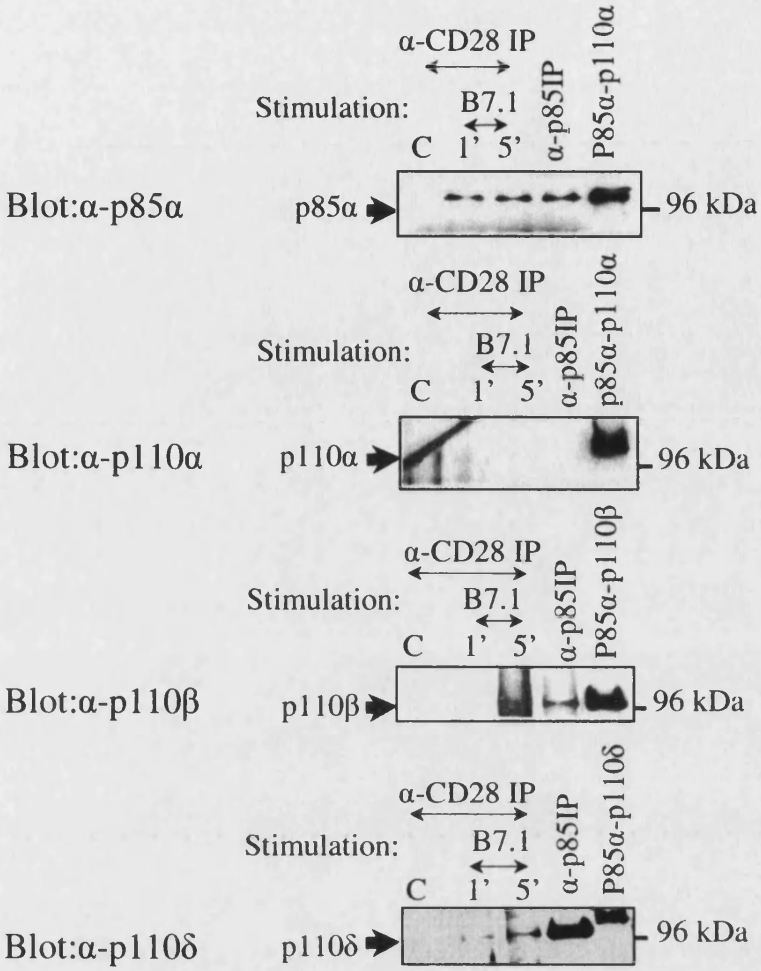


Figure 4: Association of p85α and p110 isoforms with CD28 after ligation by B7.1.

$2x10^7$ Jurkat T cells were either left un-stimulated as a control (C) or stimulated with CHO-B7.1$^+$ cells at a ratio of 3:1 respectively. Cells were lysed and subject to precipitation with 1 μg of either anti-CD28 antibody, 9.3, or anti-p85α antibody. Precipitated proteins were separated by 7.5 % SDS-PAGE, along with 0.01 μg of p85α-p110α, β, or δ recombinant protein, prior to immunoblotting with anti-p85α (top panel), anti-p110α (second panel), anti-p110β (third panel), and anti-p110δ (bottom panel) each at a concentration of 1 μg per ml in 0.05 % marvel/PBS. Proteins were visualised by chemiluminescence. These data are from a single experiment and are representative of a further four experiments.

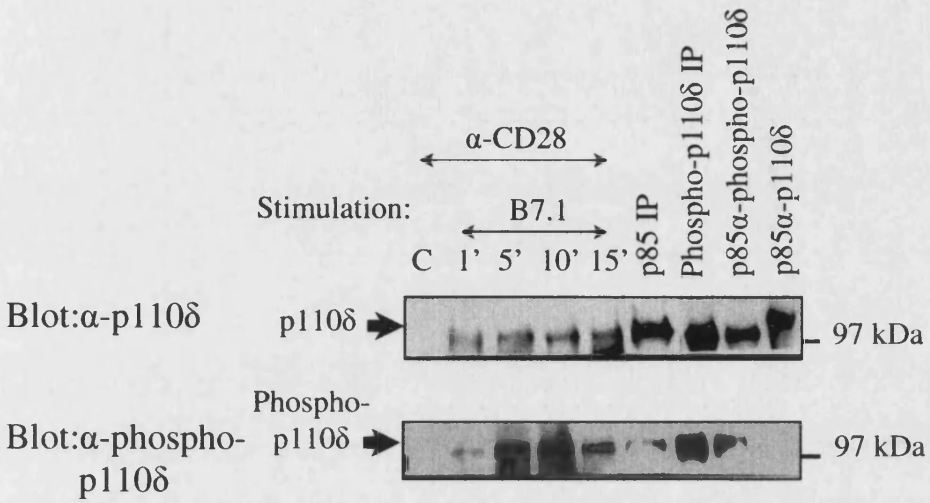


Figure 5: Association of p110δ and phospho p110δ with CD28 after ligation by B7.1.

$2x10^7$ Jurkat T cells were either stimulated with CHO-B7.1$^+$ cells at a ratio of 3:1 respectively, or left unstimulated as a control (C). Cells were lysed and subject to immunoprecipitation with 1 μg of anti-CD28 antibody 9.3. For control purposes $2x10^7$ cells were lysed and immunoprecipitated with either anti-p85 or anti phospho-p110δ, in addition control peptides representing p85α-p110δ and p85α-p110δP were electrophoresed in parallel with the described immunoprecipitates. Samples were separated by 7.5 % SDS-PAGE and transferred onto nitro-cellulose for immunoblotting with anti p110δ or anti phospho-p110δP antibody, at a concentration of 1μg pre ml in 0.05 % marvel/PBS. Proteins were visualised via chemiluminescence. These data are from a single experiment which is representative of three others.

(*top panel*, figure 5) and which was readily detectable at 1 minute and was sustained for 15 minutes, following B7.1 stimulation (*top panel*, figure 5).

DETECTION OF PHOSPHO-p110δ IN p110δ IMMUNOPRECIPITATES IN RESPONSE TO B7.1 STIMULATION.

In order to further examine the serine phosphorylation of p110δ which is brought about by B7.1 ligation, immunoblot analysis of anti-p110δ immunoprecipitates derived from resting or B7.1 stimulated Jurkat cells, using anti-p110δP and anti-p110δ antibodies, was carried out. Anti-p110δP antibodies were a kind gift from Bart Vanhasebroeck, (Ludwig Institute, London) and had been raised in rabbits immunised with a synthetic peptide (^{1028}KTKVNWLAHNVS$_P$KDNR^{1044}Q, where S_P= phospho-serine). This revealed the appearance of phospho-p110δ in anti-p110δ immunoprecipitates after B7.1 stimulation (figure 6). The kinetics of the association mirrored that observed for association of phospho-Ser p110δ with CD28, in that the marked association of phospho-p110δ could be detected at 5 minutes. However, in contrast to the co-precipitation of phospho-p110δ with CD28, phospho-p110δ could still be detected in anti-p110δ immunoprecipitates 15 minutes following B7.1 stimulation (figure 6). Recombinant p85α-p110δ and p85α-phospho-p110δ proteins were immunoblotted in parallel and demonstrated Ab specificity. Re-probing with anti-p110δ Ab demonstrated equal loading and transfer of immunoprecipitated protein (figure 6).

***IN VITRO* LIPID KINASE ACTIVITY OF CD28 ASSOCIATED p110 ISOFORMS.**

In an attempt to measure the effects of CD28 ligation on different p110 isoforms enzymatic activity, Jurkat T cells were stimulated using CHO-B7.1$^+$ cells and each p110 isoform was immunoprecipitated, and their *in vitro* lipid kinase activity was examined. Anti-p110α immunoprecipitates exhibited high basal *in vitro* lipid kinase activity which decreased upon B7.1 ligation (figure 7). In contrast anti-p110β immunoprecipitates showed low basal lipid kinase activity which was markedly increased upon CD28 ligation by B7.1. This activity was sustained for up to 5 minutes post CD28 ligation. Finally anti-p110δ immunoprecipitates displayed transient lipid kinase activity which was basally low but elevated to maximal level at 1-2 minutes post activation which had returned to basal levels within 5 minutes. As a positive control the lipid kinase activity associated with anti-CD28 immunoprecipitates from CHO-B7.1$^+$

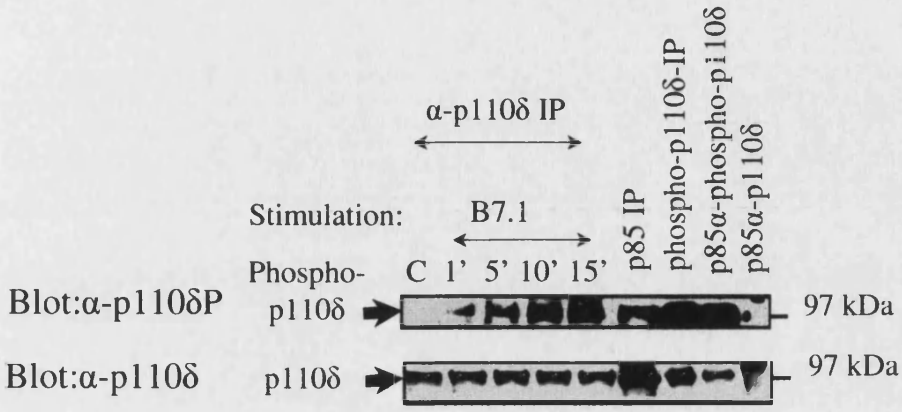


Figure 6: Appearance of phospho-p110δ in p110δ immunoprecipitates after CD28 ligation by B7.1.

$2x10^7$ Jurkat cells were either stimulated with B7.1expressing CHO cells or left unstimulated as a control (C). Cells were lysed and immuno-precipitated with 1 μg anti-p110δ antibody. As a control $2x10^7$ Jurkats were lysed and immuno-precipitated with 1 μg anti-p85 or anti-p110δ antibodies, and electrophoresed with the above precipitates and control peptides, representing, p85α-phospho-110δ, and p85α-p110δ. Samples were separated by 7.5 % SDS-PAGE and transferred onto nitro cellulose for immunoblotting with anti-phospho-p110δ at a concentration of 1 μg per ml in 0.05 % marvel/PBS. Blots were then stripped and re-probed with anti-p110δ antibody at 1 μg per ml in 0.05 % marvel/PBS, to verify equal loading of immunoprecipitated protein (bottom panel). Proteins were visualised via chemiluminescence. These data are from a single experiment and are representative of three other experiments.

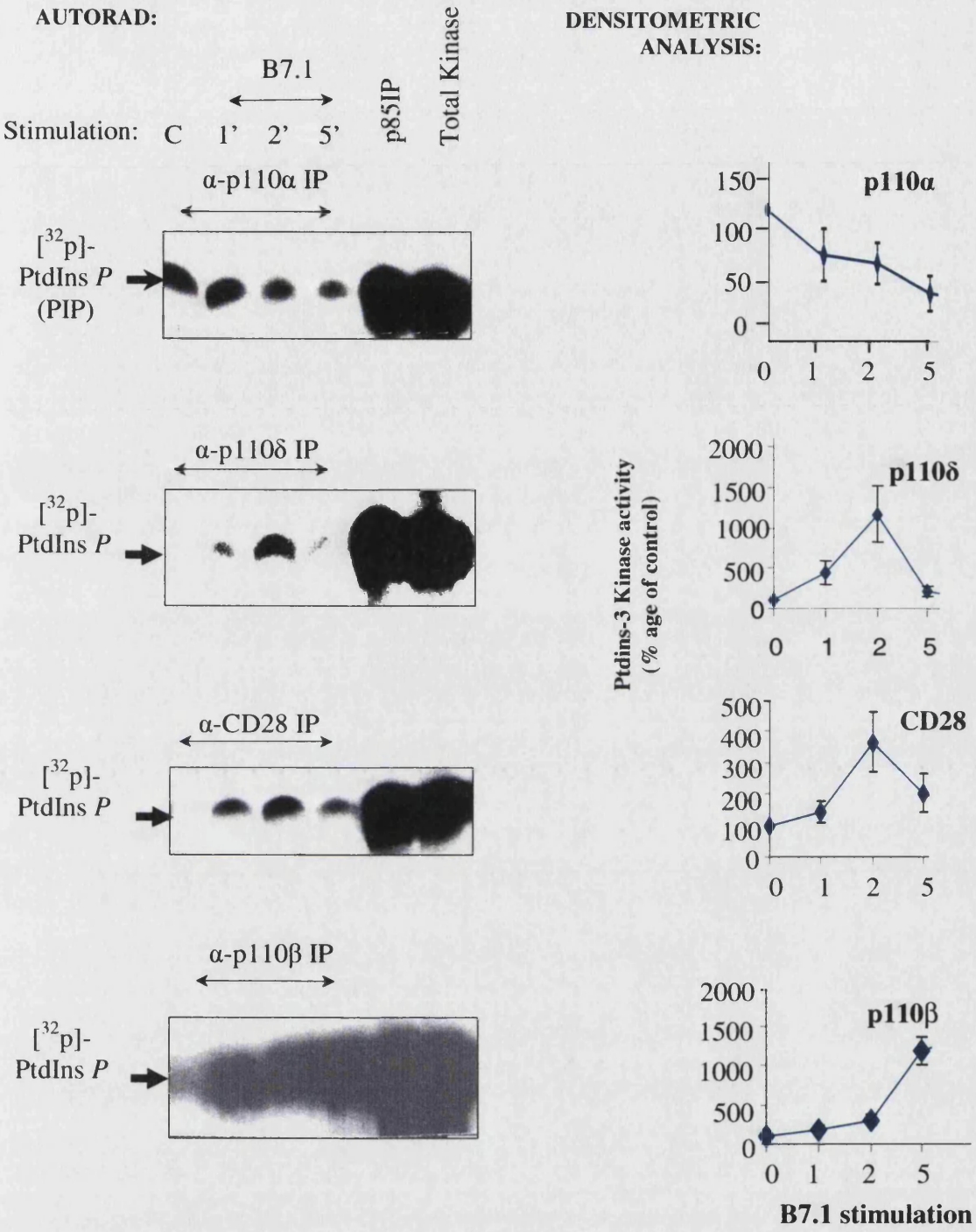


Figure 7: Comparison of p110α, p110β p110δ and CD28 associated vitro lipid kinase activity.

$2x10^7$ Jurkat cells were either left unstimulated as a control (C), or stimulated with CHO-B7.1$^+$ cells, at a ratio of 3:1 respectively. Cells were lysed and immunoprecipitated with 1 μg of either anti-CD28 mab 9.3, or anti-p110 α, β and δ. Immunoprecipitates were then incubated with [^{32}P]-γATP, and phosphatidylinositol, in lipid kinase buffer, at 37°C for 20 mins (as detailed in Materials and Methods. Resultant [^{32}P]-phoshphatidylinositol lipids were separated by TLC, and visualised by autoradiography (left hand panels). Autoradiograph data shown are from a single experiment and are representative of four experiments. Autoradiographs were subject to densitometric analysis, and data values representing lipid kinase activity attributable to CD28, p110α, β, or δ immunoprecipitates was expressed as a percentage of lipid kinase activity immunoprecipitated from resting cells (% of control). The error bars represent the mean ± SEM for four separate experiments (Right hand panels).

stimulated Jurkats was assessed and revealed an increase in lipid kinase activity after ligation of CD28. The lipid kinase activity present in p85 immunoprecipitates and whole cell lysates is also indicated (figure 7).

EFFECTS OF OKADAIC ACID AND WORTMANNIN TREATMENT ON p110δ PHOSPHORYLATION *IN VITRO*

One interpretation of the data outlined in the above experiments is that CD28 can stimulate the autophosphorylation of p110δ, through activation of p110δ's intrinsic serine kinase activity which elicits a regulatory auto-phosphorylation of the p110δ C-terminus. In order to verify that the protein kinase activity measured in this assay was attributable to PI3K kinase activity and not to a contaminating protein kinase, the sensitivity of the phosphorylation of p110δ to the PI3K inhibitors LY249002 and wortmannin and measured. Given that PI3K's exhibit dual specificity, as both lipid and protein kinases, it was necessary to verify that was sensitive to PI3K inhibitors.

Initially, *in vitro* kinase assays were carried out using immunoprecipitates derived from CHO-B7.1 stimulated Jurkat T cells. However as these preliminary assays were unsuccessful, immunoprecipitates derived from the hCD28 transfected T cell hybridoma DC27.1 were employed. It was observed that the *in vitro* phosphorylation of a 110 kDa protein associated with anti-p110δ immunoprecipitates was basally elevated in non stimulated cells. The phosphorylation of this band was further elevated upon CHO-B7.1$^+$ stimulation and was maximal at five minutes (figure 8). In addition to the 110 kDa band, phosphorylation of further bands of approximately 59 kDa, 56 kDa, and 52 kDa could be seen in immunoprecipitates from resting cells, which was slightly elevated following CHO-B7.1$^+$ cell stimulation. Furthermore, it was demonstrated that the *in vitro* kinase activity associated with p110δ immunoprecipitates from B7.1 stimulated DC27.1 CD28$^+$ T cells, could be abrogated by the PI3K inhibitor wortmannin. Hence the 100, 59 56, and 52 kDa phospho proteins described above, were completely absent following wortmannin treatment (Figure 8). Cells were also treated in parallel with okadaic acid, a Ser/Thr phosphatase protein I and protein phosphatase IIa inhibitor, which demonstrated that inhibition of basally active serine phosphatases allowed an increase in levels of p110δ phosphorylation in resting DC27.1 CD28$^+$ T cells (see figure 8). Finally, as a positive control, kinase activity associated with anti-p85α immunoprecipitated proteins

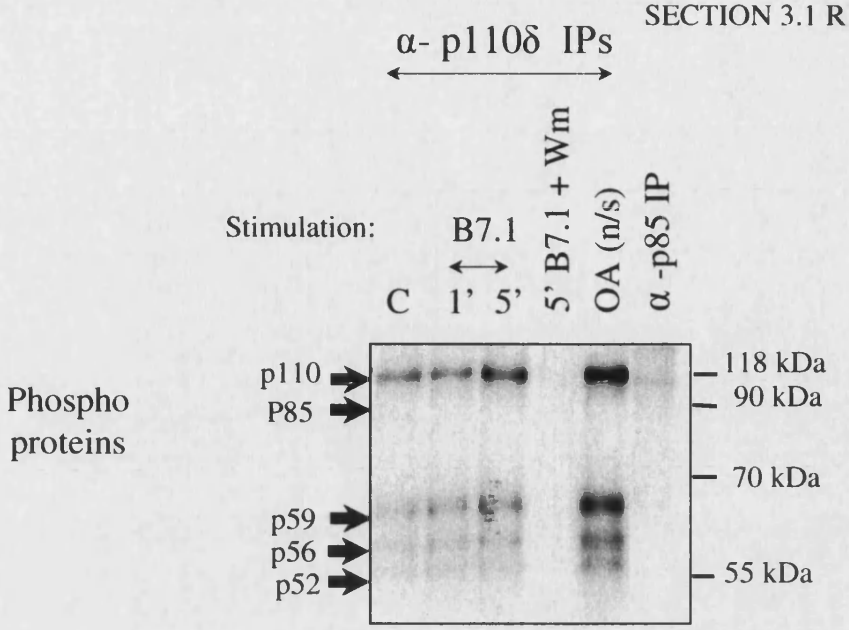


Figure 8: Effect of wortmannin and okadaic acid treatment on CD28 mediated phosphorylation of p110δ and *In Vitro* kinase activity associated with p110δ- immunoprecipitated protein complexes.

$2x10^7$ DC27.1 $CD28^+$ T cell hybridoma cells were either left untreated (C) or stimulated with CHO-$B7.1^+$ cells at a ratio of 3:1 respectively, and then lysed.. To analyse the effects of wortmannin and okadaic acid pre-treatment on *in vitro* phosphorylation of p110δ, $2x10^7$ cells were either treated with 50 nM wortmannin (Wm), prior to stimulation, or treated with 10 nM okadaic acid (OA) left unstimulated. Samples were immunoprecipitated with 1 μg anti-p110δ, or anti-p85α as a positive control and assayed for *in vitro* kinase activity, by incubation with [^{32}P]-ATP, and Na^{2+}ATP in kinase buffer. Nascent [^{32}P]-labelled proteins were separated via SDS-PAGE on a 12cm, 10% gel, and visualised by autoradiography. These data are from a single experiment and are representative of two other experiments.

were examined, and the presence of a 110 kDa band, and a fainter 85 kDa band reflected the basal phosphorylation state of p85 and 110 isoforms in this cell line.

SUMMARY

1. High levels of the PI3K product PI(3,4,5)P_3 were basally present in unstimulated Jurkat T cells.
2. In accordance with previous studies, ligation of the CD28 co-receptor by B7.1 led to a rapid elevation of PI(3,4,5)P_3 levels, which was maximal at ten minutes.
3. Expression of PI3K catalytic isoforms p110α, p110β, and the novel leukocyte expressed catalytic isoform, p110δ can be detected in jurkat T cells. p110δ protein was also detected in freshly isolated human T lymphocytes and cultured human T lymphoblasts, consistent with the leukocyte restricted distribution of this catalytic isoform.
4. B7.1 stimulation of Jurkats, led to the co-association of p110β and p110δ with the CD28 coreceptor. The consistent detection of p110α in CD28 immunoprecipitates was not possible.
5. Auto-phosphorylation of p110δ occurs in response to CD28 ligation by B7.1, and is maximal at 10 minutes but diminishes at fifteen minutes post stimulation.
6. B7.1 ligation induces phosphorylation of the total cellular pool of p110δ, which occurs rapidly and is sustained at 15 minutes.
7. B7.1 ligation of CD28 regulates the intrinsic lipid kinase activity associated with immunoprecipitates of PI3K isoforms, p110α p110β and p110δ, measured *in vitro*. Lipid kinase activity associated with anti-p110α IPs is abrogated following CD28 ligation. Anti-p110β IPs exhibit elevated lipid kinase activity upon B7.1 ligation which is sustained at 5 minutes. Anti-p110δ IPs exhibit initial lipid kinase activity, however this is abrogated after 5 minutes, which correlates with the serine phosphorylation of p110 δ observed by western blotting.
8. p110δ exhibits a basal level of phosphorylation which is elevated upon B7.1 ligation, as detected via an *in vitro* kinase assay. Phosphorylation of p110δ is abrogated in vitro by pre-treatment of cells with the PI3K inhibitor wortmannin prior to B7.1 stimulation. Treatment of cells with okadaic acid dramatically enhances basal levels of p110d phosphorylation. (Phosphoproteins of 59,56,

and 52 kDa can also be detected in association with p110δ, and the phosphorylation of these proteins in response to CD28 stimulation is observed and can be inhibited by wortmannin. Furthermore okadaic acid treatment of cells enhances the basal phosphorylation of these unidentified proteins).

3.1 DISCUSSION - CD28 AND PI3K

The CD28 mediated activation of PI3K, and the concomitant production of D-3 phosphoinositides, has been extensively described in T cells (Ward et al 1993) and occurs at a 5-10 fold greater magnitude than activation of PI3K via the TCR (Ward et al 1992). In particular, the accumulation of the rare phosphoinositide PI(3,4,5)P_3 in response to receptor ligation, may represent a critical upstream component of a co-stimulatory signalling pathway that is driven by the CD28 co-receptor. However, the role of PI3K in CD28 mediated costimulation is very controversial; Pharmacological and genetic studies have presented conflicting evidence as to the importance of PI3K in T cell costimulation. Studies in which truncation mutants of the CD28 cytoplasmic tail were expressed in T cell hybridoma cells, suggested that PI3K association with tyrosine residues within the CD28 tail was not essential for efficient costimulation (Nagel et al 2000). Earlier studies in Jurkat T cells demonstrated that wortmannin inhibition was not sufficient to inhibit IL-2 production and abolition of tyr 170 of the CD28 tail, which prevents PI3K association, did not inhibit CD28 mediated costimulatory signals leading to IL-2 production (Truitt et al 1995, Lu et al 1995). Furthermore T cells from p85 α knockout mice do not exhibit impaired T cell responsiveness, data which further contributes to the controversy surrounding PI3K's relationship to CD28 (Suzuki et al 1999, Fruhman et al 1999). This study therefore sought to further investigate the nature of the interaction between CD28 and PI3K.

CD28 MEDIATED PI(3,4,5)P_3 ACCUMULATION

This study has described *in* vitro data which indicates that the CD28 driven accumulation of PI(3,4,5)P_3, observed by this and previous studies (Ward et al 1993) to occur in Jurkats, may be largely attributable to the p110β catalytic isoform of PI3K. The rapid and sustained stimulation of p110 β *in vitro* lipid kinase activity, seen in this study upon CD28 ligation, closely correlates with B7.1 induced PI(3,4,5)P_3 accumulation in intact cells. In contrast, CD28 is shown here to mediate the negative regulation of p110α and p110δ lipid kinase activity. Thus this study implicates p110β as the major catalytic isoform which acts to couple CD28 to a diverse array of intracellular pathways, that culminate in IL-2 production (Ward et al 1995), T cell proliferation (Karnitz et al 1995) and cell survival (Parry et al 1997). However, the physiologically

relevant regulatory mechanisms involved in PI3K activation *in vivo* may not be wholly represented by *in vitro* analysis of lipid kinase activity, and as such these data can only be taken as an indication of the CD28 mediated regulation of different PI3K isoforms. For example the existence of 4- and 5- lipid kinases, which may contaminate immuno-precipitated PI3Ks, could also contribute to the [32]-P labelled PI-*P* detected by a lipid kinase assay.

P110δ PROTEIN KINASE ACTIVITY

The identification of p110δ, a newly characterised p110 isoform specifically expressed in leukocytes, has raised questions regarding the physiological role of this isoform, which up until now has been predominantly studied *in vitro* (Vanhaesebroeck et al 1997). Furthermore, the observation that p110δ displays auto-serine phosphorylation capacity which has been described to occur *in vitro* and to minimal levels in B cells and mast cells (Vanhaesebroeck et al 1997) has led to the use of p110δ as a working model by which the protein kinase activity of PI3Ks can be studied. The catalytic domains of all PI3Ks share homology with the kinase domain of protein kinases, and this observation led to the identification of lipid and protein kinase activities for class I and III PI3Ks (Hunter et al 1995).

This study has demonstrated that the intrinsic protein kinase activity of p110δ is activated in response to CD28 ligation, and targets serine residue ^{1039} within the p110δ catalytic domain. Not only does this novel observation suggest that CD28 mediates the regulation of a set of functionally distinct PI3K catalytic isoforms, but it has also led to the suggestion that CD28 may target an as yet unidentified array of protein targets via p110δ's protein kinase activity. The identification of physiological substrates for the class I and III PI3K protein kinase activities has been limited and most PI3K protein kinase activities that have been demonstrated either autophosphorylate their catalytic subunits (Stoyanova et al 1997) or target the associated adaptor subunits (Carpenter et al 1993). One exception is the class1A PI3K p110α which has been putatively described to phosphorylate the insulin receptor substrate, IRS-1 (Lam et al 1994, Uddin et al 1997). In addition to the autophosphorylation capacity of p110δ, the class 1B PI3K, p110γ has intrinsic autophosphorylation capacity. In contrast to p110δ, however, p110γ's protein kinase activity has not been associated with the negative regulation of its

lipid kinase activity (Stoyanova et al 1997). However, p110α protein kinase activity can impart negative regulation of its own lipid kinase activity through the serine phosphorylation of p85α (Carpenter et al 1993). Whilst this study did not examine the phosphorylation of p85α in p85/p110α heterodimers, or conclusively demonstrate a co-association between p85 and p110α, it is interesting to note that CD28 stimulated a marked down regulation of the constitutive lipid kinase activity that could be immunoprecipitated via p110α. Hence, lipid kinase activity attributable to p110α may also be negatively regulated by CD28 ligation. Conjecture could surmise that the CD28 driven negative regulation of p110α lipid kinase activity is conferred via the serine phosphorylation of p85α by p110α.

It is therefore unclear whether in addition to autophosphorylation of p110δ, the CD28 mediated activation of p110δ protein kinase activity targets unidentified protein substrates. The existence of unique biochemical targets for p110δ's protein kinase activity would parallel the accumulating evidence which describes the role of the PI3K like kinase domain of the PIK related proteins (mTOR, ATM) in mediating *in vivo* protein phosphorylation of a mounting array of substrates (Brunn et al 1997, Shieh et al 1997, Canman et al 1998). Further evidence that PI3K protein kinase activity can target exogenous protein substrates has arisen from studies in which protein kinase only p110γ mutants were demonstrated to direct the phosphorylation of MEK1 (Bondeva et al 1998).

p110δ - LIPID KINASE ACTIVITY

This study has illustrated p110δ 's role in a physiologically relevant signalling cascade. The observed serine phosphorylation of p110δ described above correlates with the CD28 mediated negative regulation of p110δ's lipid kinase activity. This observation may support previous work which suggests that **auto** phosphorylation of p110δ elicits the inhibition of p110δs lipid kinase activity (Vanhaesebroeck et al 1999). Structural analysis suggests that the phosphorylation of Serine^{1039}, which would create a negative charge in the C-terminal region of p110δ surrounding this residue, may be sufficient to abrogate the binding of lipid substrates as this region has been implicated in binding to inositol head groups (Vanhaesebroeck et al 1999). Indeed the artificial creation of a

negative charge at serine 1039 (D-E) of p110 δ correlates with a reduction in lipid kinase activity (Vanhaesebroeck et al 1999).

The current study has demonstrated that the lipid kinase activity of p110δ may be negatively regulated following CD28 ligation by p110δ's intrinsic protein kinase activity which mediates phosphorylation of serine [1039]:

1) B7.1 stimulation of Jurkat cells mediates the down regulation of anti-p110δ immunoprecipitates *in vitro* lipid kinase activity.
2) Data gained through the immunoblotting of anti-p110δ immunoprecipitates suggests that p110δ is serine phosphorylated in response to CD28 ligation.
3) *In vitro* data indicates that p110δ is subject to protein phosphorylation following B7.1 stimulation of CD28 expressing T cell hybridoma stimulation, and that this phosphorylation event is mediated in a PI3K dependent fashion.

The role of CD28 mediated negative regulation of the p110δs lipid kinase activity is not obvious.

Perhaps significantly, the co-association of p110δ with CD28 is sustained over the time courses examined in this study, and may even increase nominally. The attenuation of p110δ's briefly elevated lipid kinase activity occurs shortly after the observed co-association of CD28 and p110δ. Thus, in addition to the speculation that p110 δ may elicit the CD28 driven serine phosphorylation of as yet unidentified proteins, p110δ could be considered to play a role in negatively regulating lipid accumulation following CD28 ligation. The sustained association of quiescent p85/p110δ heterodimers with CD28 dimeric receptors would preclude the co-association of operational PI3K isoforms, namely p85/p110β, with the p85 SH2 interaction motifs within the CD28 cytoplasmic tail. Alternatively heterogeneity in the coupling of p110 isoforms to a single CD28 dimer may exist. Thus p110δ may play a role in dampening PI(3,4,5)P_3 production, through the competitive inhibition of CD28/p110β associations.

The physiological significance of a role for p110δ in down regulating the cell's potential PI(3,4,5)P_3 production is not easily determined. *In vitro* data suggests that p110βs co-

association with CD28 may be sufficient to lead to the D-3 phosphoinositide production observed following CD28 ligation in intact cells. Furthermore, at later time points, serine phosphorylation of p110δ associated with CD28 appears to diminish and this may correlate with a re-elevation of its lipid kinase activity. Thus, it may be that p110δ plays a minor role in dampening the cells D-3 phosphoinositide production as the considerable association of p110β protein with CD28 has been detected and is shown to be sustained for 15-20 mins following receptor ligation. Furthermore, CD28 mediated PI(3,4,5)P_3 production has been described here and in previous studies (Ward et al 1996) to be dramatic, and reflects the sustained *in vitro* lipid kinase activity attributed to p110β and the co-association of p110β with CD28.

Later in this study the high basal activity of PI3K apparent in Jurkat T cells, which may be a characteristic feature of leukaemic cell lines, will be discussed. In the light of this data it could be considered that the significance of a regulatory mechanism controlled by p110δ may vary between cell lines. This may possibly explain the difficulty experienced in detecting the *in vitro* phosphorylation of p110δ described in Jurkat T cells, and the need to use an alternative cell line for these assays.

The relevance of a role for p110δ in regulating PI(3,4,5)P_3 accumulation following CD28 ligation could also be determined through the measurement of phosphoinositide production in response to receptor ligation in p110δ deficient cells. Moreover, if the size of the cellular pool of p110δ is too small to be considered to play an active role in regulating global cellullar levels of PI(3,4,5)P_3, it may function in the regulation of compartmentalised PI(3,4,5)P_3 production. The early lipid kinase activity of p110δ may act to drive pathways activated soon after receptor ligation, which are subject to negative regulation at later time points. The transient re-distribution of PKB to the membrane, that has been observed following BCR ligation (Astoul et al 1999) but is described to rapidly re-localise back to the cytosol and nucleus at time points when global PI(3,4,5)P_3 production is still high, could be regulated by compartmentalised pockets of PI(3,4,5)P_3 production mediated by a distinctly regulated isoform of PI3K. The down regulation of p110δ lipid kinase activity may have important implications *in vivo*, for example by supressing the anti-apoptotic action of PI3Ks in cancer cells (Franke et al 1997).

This study has also shown that the treatment of Jurkat T cells with the PP1 and PP2a family phosphatase inhibitor, okadaic acid, enhances levels of p110δ *in vitro* phosphorylation in resting cells, suggesting that p110δ may be a target for serine phosphatase activity. This observation is in accordance with prior data derived from the immunoblot analysis of okadiac acid treated Jurkat T cells with anti-phospho serine^{1039} p110δ antibodies (Vanhaesebroeck et al 1999). It could be proposed that the actions of such serine phosphatases negatively regulate levels of serine phosphorylated p110δ and thus allow the accumulation of a pool of non-phosphorylated p110δ which can contribute to the accumulation of D-3 phosphoinositides in a resting cell, and upon initial receptor ligation, whilst receptor induced autophosphorylation of p110δ inhibits lipid kinase activity at later time points. The transient accumulation of [^{32}-P] labelled phosphoinositides, observed via the assay of *in vitro* lipid kinase activity of p110δ immunoprecipitates, supports this model.

SECTION 3.1- CONCLUSIONS

The recruitment of different catalytic isoforms of PI3K to CD28 upon ligation by its physiological ligand B7.1, adds further support to the previously identified role of PI3K in CD28 mediated signalling pathways. Furthermore the differential regulation of the *in vitro* lipid kinase activity attributable to each of these isoforms, upon B7.1 stimulation, suggested that the recruitment of different PI3K heterodimers to CD28 may be of functional importance. Also of particular interest is the activation of p110δ's auto serine phosphorylation capacity by CD28, and the possibility of the existence of additional protein kinase substrates that this raises.

Through the existence of multiple catalytic isoforms, PI3K may be considered to gain biochemical access to the diverse array of functional outcomes mediated by CD28 regulated PI3K dependent signalling cascades (see diagram 15). Equally, the differential regulation of these isoforms, as demonstrated in this study, upon receipt of a single extra-cellular stimulus, may facilitate the CD28 mediated control of multiple intracellular pathways. Thus, the bifurcation of PI3K's biochemical impact through lipid and protein kinase activites could further augment the capacity of PI3K to influence signal transduction.

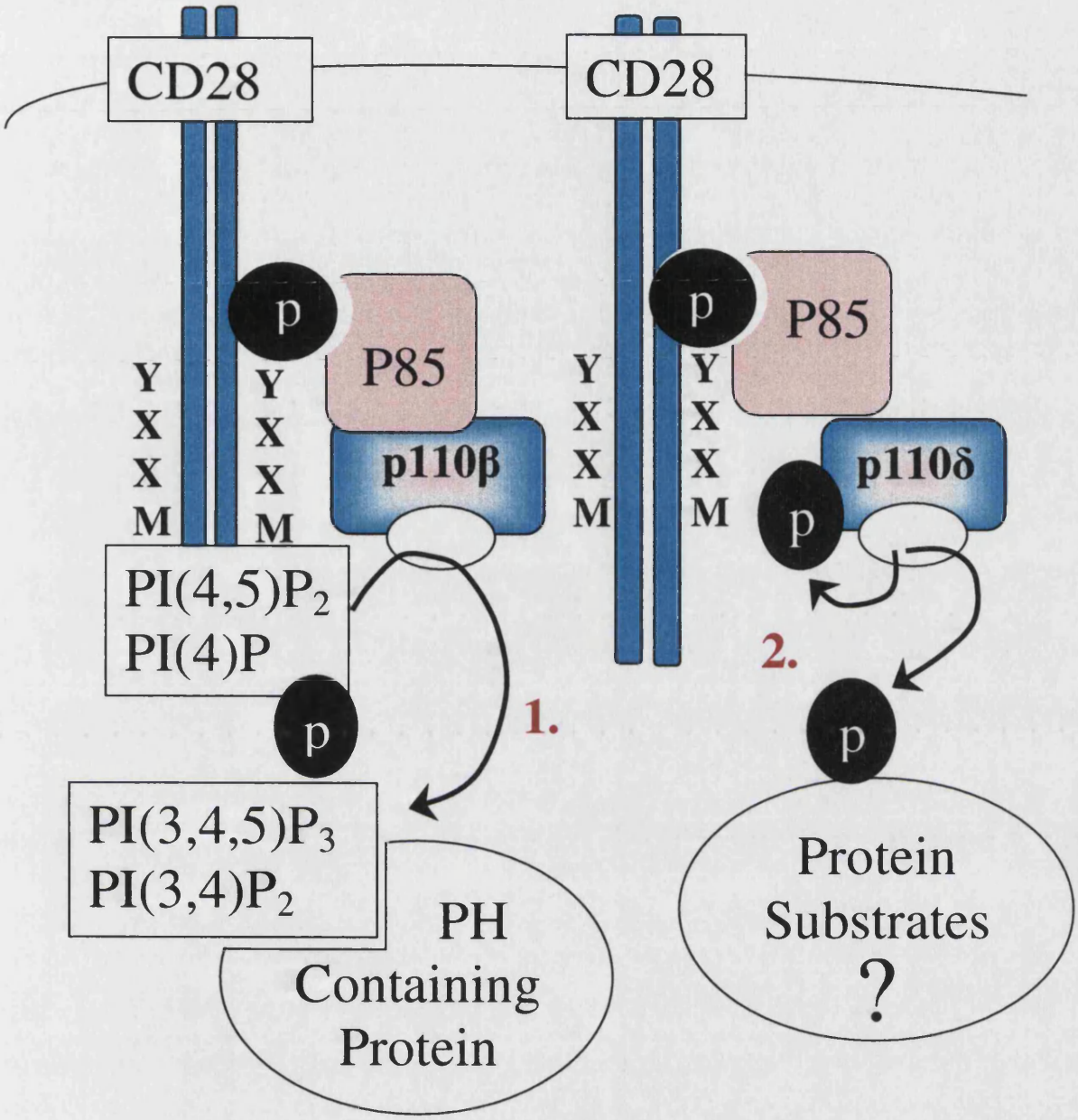


Diagram 15: Schematic representation of p110δ/p85 and p110β/p85 heterodimers co-association with CD28.

Following CD28 stimulation by B7.1 in Jurkats the co-association of p110β and p110δ has been observed to occur with CD28. The potential functional outcomes of these co-associations are depicted above. (1.) This study has indicated that the predominant production of $PI(3,4,5)P_3$ may occur following p110β/p85 heterodimer co-association with CD28. Therefore, p110β may be responsible for the recruitment of PH domain containing proteins, such as PKB, to the membrane, for subsequent activation by membrane proximal protein kinases. (2.) p110δ, meanwhile, may undergo an autophosphorylation event upon CD28 ligation through its intrinsic protein kinase activity, which down regulates its lipid kinase activity leading to the abrogation of $PI(3,4,5)P_3$ production. Whether additional protein kinase substrates for p110δ exist in T cells is not clear at present, but if such proteins exist they may represent further targets for CD28 mediated biochemical signals.

3.2 SHIP

3.2.1 SHIP AND CD28

The 145 kDa SH2 containing 5-polyphosphatase, SHIP has been implicated in the degradation of the PI3K product, PI(3,4,5)P_3, and hence regulation of PI(3,4,5)P_3 driven effector pathways, via conversion to PI(3,4)P_2 (Damen et al 1996). Given that CD28 ligation results in the major accumulation of PI(3,4,5)P_3 (Ward et al 1995) this study aimed to determine whether SHIP is coupled to and/or regulated by CD28.

The murine T cell hybridoma DC27.1 clone DWT6.11, which is a human CD28 (hCD28) stable transfectant (Pages et al 1996), has been previously demonstrated to express considerable levels of 145 kDa SHIP (Edmunds et al 1999). This cell line was therefore chosen as a model in which to study the effects of CD28 ligation on SHIP tyrosine phosphorylation. Ligation of CD28 in this cell line was achieved with the anti-CD28 mAb, 9.3, which recognises human CD28. The physiological ligand for CD28, B7.1, was not the tool of choice in this model since one might expect murine expressed CD28 to contribute to the biochemical cascade. However cytometric analysis of hCD28 versus mCD28 expression has demonstrated approximately 50 fold higher levels of hCD28 (personal communication, Steven Burgess).

CD28 SPECIFICALLY MEDIATES TYROSINE PHOSPHORYLATION OF SHIP.

In unstimulated DC27.1 cells, there is no apparent basal phosphorylaton of SHIP detectable in anti-SHIP immunoprecipitates (figure 9). Ligation of hCD28 in this cell line, with the anti-human CD28 monoclonal antibody 9.3, results in the rapid tyrosine phosphorylation of 145 kDa SHIP after 1 minute of stimulation, and this is sustained for up to 10 minutes following ligation (figure 9).

Several groups have reported the tyrosine phosphorylation of SHIP in response to FCγRIIB engagement in B cells (Chacko et al 1996). It was therefore important to establish that the observed tyrosine phosphorylation of SHIP after CD28 ligation was not due to any FCγRIIB association with the FC region of the 9.3 mAb. Several lines of evidence indicate that FCγRIIB ligation does not occur. Firstly, the control isotype matched (figure 9) IgG2a did not induce tyrosine phosphorylation of SHIP in anti-SHIP immunoprecipitates. Secondly, it was established that SHIP tyrosine phosphorylation was not mediated through FCγRIIB binding using the FCγRII/III blocking antibody,

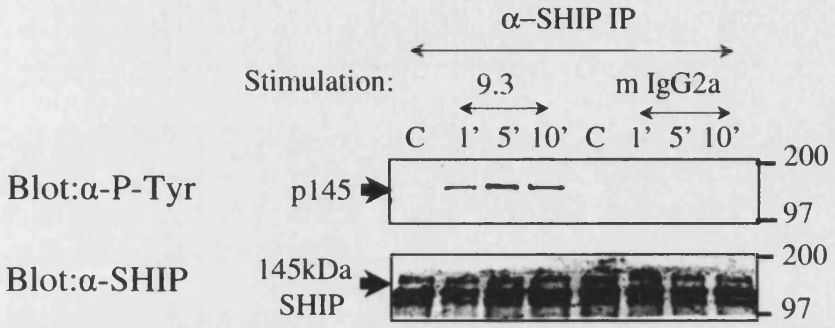


Figure 9: Tyrosine phosphorylation of SHIP following CD28 ligation with anti-CD28 antibody 9.3.

$2x10^7$ DC27.1 $hCD28^+$ T cell hybridoma cells were either left unstimulated as a control (C), or stimulated with 5 μg either anti CD28 antibody, 9.3, or with an isotype matched mouse IgG 2a antibody. Cells were lysed and subject to immunoprecipitation with 1μg anti-SHIP polyclonal antibody. Precipitated proteins were separated via 7.5% SDS-PAGE and transferred onto nitro-cellulose for immunoblotting with anti-phospho tyrosine antibody, 4G10, at a concentration of 1μg per ml in 0.05% marvel/PBS. Blots were subsequently stripped and re-probed with anti-SHIPAb at 1μg per ml in 0.05% marvel/PBS, to verify equal loading of immunoprecipitated protein. Proteins were visualised via chemiluminescence. These data are from a single experiment and are representative of three other experiments.

2.4G2. This antibody blocks non-antigen specific binding of immunoglobulins to the mouse FCγRII and FCγRIII. Pre-treating cells with this antibody had no effect on the levels of SHIP tyrosine phosphorylation observed following hCD28 stimulation via mAb 9.3 in anti-SHIP immunoprecipitates (figure 10). Finally the ligation of CD28 via specific ligand B7.1, on CHO-B7.1$^+$ cells, also stimulated the strong tyrosine phosphorylation of SHIP (Figure 11). It should be noted that under these conditions, tyrosine phosphorylation may result from the interaction of B7.1 with both the transfected hCD28 and naturally expressed murine CD28, although levels of murine CD28 are approximately 50 fold lower than that of transfected hCD28. The CHO cells on which B7.1 is presented to the hybridoma T cell line do not express detectable levels of SHIP, so it is unlikely that CHO cells contribute any of the tyrosine phosphorylated SHIP that is detected following CD28 ligation via B7.1 (figure 11). Each immunoblot was stripped and re-probed with polyclonal SHIP anti-serum, to confirm efficiency of immunoprecipitation and transfer onto nitro-cellulose membranes (figures 9, 10, and 11).

CO-STIMULATORY SIGNALS FURTHER ENHANCE PHOSPHORYLATION OF SHIP.

Ligation of the TCR has been reported to stimulate the tyrosine phosphorylation of SHIP (Lamkin et al 1997). Given the regulatory role of CD28 on TCR-mediated T cell activation, it was important to compare the phosphorylation/activation of SHIP in response to ligation of CD28 and TCR either alone or in combination. Hence, ligation of hCD28 with mAb 9.3 or naturally expressed murine CD3 with the 2C11 Ab, both stimulated the strong tyrosine phosphorylation of SHIP to similar degrees, although the CD3 response was initilally stronger and appeared to be more transient in nature (Figure 12). Together these mAb were seen to stimulate an additive effect on enhanced levels of SHIP tyrosine phosphorylataion when compared to the levels observed in response to individual mAb stimulation (figure 12).

TYROSINE PHOSPHORYLATION AND ACTIVATION OF SHIP CORRELATE WITH SUB-CELLULAR RE-DISTRIBUTION UPON CD28 LIGATION.

The tyrosine phosphorylation and increased activity of SHIP in response to CD28 ligation correlated with a marked change in the cellular distribution of SHIP when

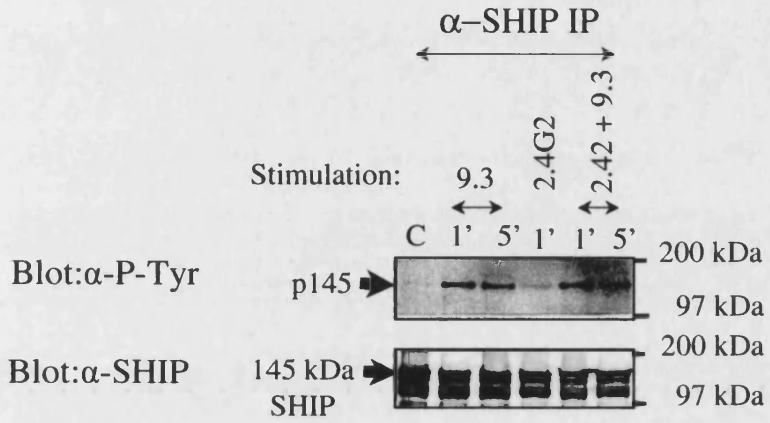


Figure 10: Tyrosine phosphorylation of SHIP in response to CD28 stimulation, in the presence of FCγRIII/II blocking antibody, 2.4G2.

$2x10^7$ DC27.1 $hCD28^+$ T cell hybridoma cells were either left unstimulated as a control (C), stimulated with 5 μg anti-hCD28 monoclonal antibody, 9.3, or treated with 5 μg anti FCγRIII/II antibody, 2.4G2 alone or with 9.3 antibody. Cells were lysed and immuno-precipitated with 1 μg anti-SHIP polyclonal anti-serum. Precipitated proteins were separated by SDS-PAGE and transferred onto nitro-cellulose for immunoblotting with anti-phosphotyrosine antibody 4G10, at a concentration of 1 μg per ml in 0.05% marvel/PBS. Blots were subsequently stripped and re-probed with anti-SHIP polyclonal anti-serum at a concentration of 1 μg per ml 0.05% marvel/PBS, to verify equal loading of immunoprecipitated proteins. Proteins were visualised by chemiluminescence. These data are from a single experiment and are representative of three separate experiments.

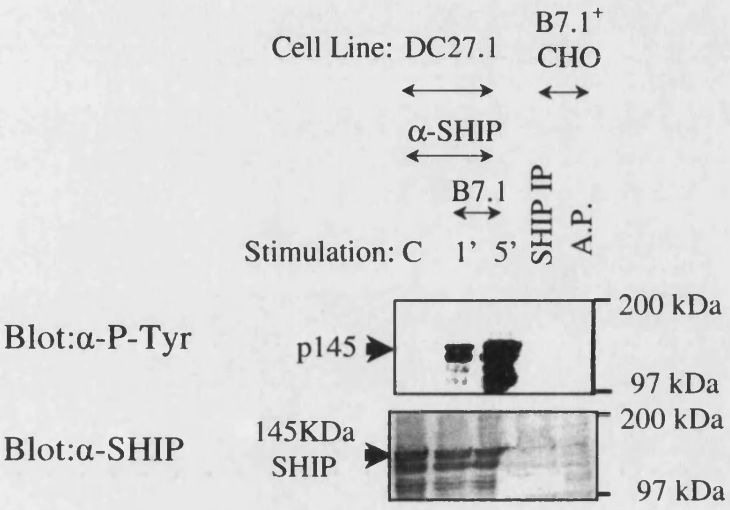


Figure 11: B7.1 ligation of CD28 stimulates 145 kDa SHIP phosphorylation.

$2x10^7$ DC27.1, CD28 transfectant T cell hybridoma cells, were either left unstimulated as a control (C), or stimulated with CHO-B7.1$^+$ at a ratio of 3:1 cells per point. Cells were lysed and immunoprecipitated with 1 μg anti SHIP polyclonal antiserum. As a control for SHIP expression $2x10^7$ CHO- B7.1$^+$ cells were lysed and proteins either acetone precipitated (A.P.) or immunoprecipitated with 1 μg anti SHIP antiserum. All proteins were separated by 7.5 % SDS-PAGE and transferred onto nitro-cellulose for immunoblotting with anti-phospho-tyrosine antibody 4G10 at a concentration of 1 μg per ml in 0.05 % marvel/PBS. Blots were subsequently stripped and re-probed with anti-SHIP polyclonal anti-serum to verify equal loading of SHIP proteins. Proteins were visualised via chemiluminescence. These data are from a single experiment and are representative of two other experiments.

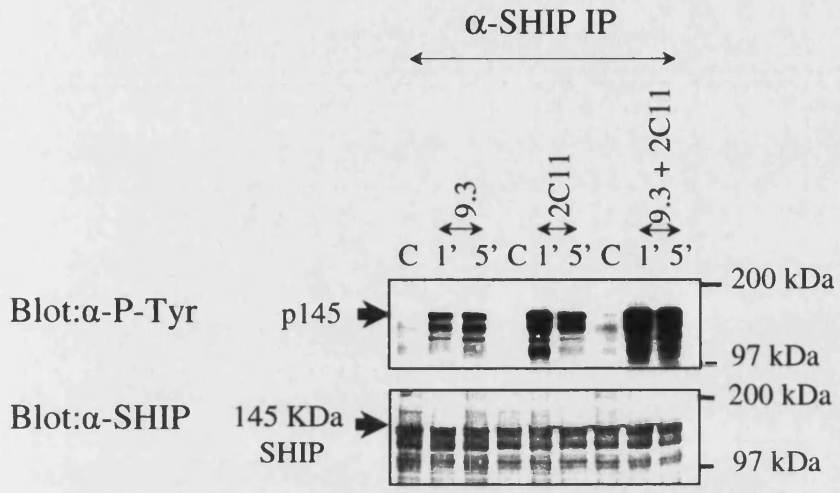


Figure 12: Tyrosine phosphorylation of SHIP in response to CD28 ligation, CD3 ligation, and co-ligation of CD3 and CD28.

$2x10^7$ DC27.1, CD28 transfected T cell hybridoma cells were either left unstimulated (C) or stimulated with 5 μg anti-CD28 antibody, 9.3, 5 μg anti-CD3 antibody, 2C11, or co-stimulated with 5 μg each of 9.3 and CD28. $2x10^7$ cells were lysed per point and immunoprecipitated with 1 μg anti-SHIP polyclonal antiserum. Precipitated proteins were separated via 7.5 % SDS-PAGE and transferred onto nitro-cellulose for immunoblotting with anti-phospho tyrosine antibody 4G10 at a concentration of 1 μg in 0.05% marvel/PBS. Blots were subsequently stripped and re-probed with anti-SHIP to verify equal loading of immunoprecipitated protein. Proteins were visualised via chemiluminescence. These data are from a single experiment which is representative of two other experiments.

analysed by sub cellular fractionation and immuno-fluorescence microscopy (A figure 13). Subcellular fractionation of resting and stimulated DC27.1 cells was carried out in order to investigate the effects of CD28 ligation on the cellular redistribution of 145 kDa SHIP protein. Fractionation of unstimulated cells demonstrated that the predominant proportion of SHIP protein resided in the cytoplasmic fraction of resting cells. However upon CD28 ligation by CHO-B7.1^{+} cells, a redistribution of SHIP protein from cytoplasmic fractions into membrane fractions was observed. Visualisation of SHIP distribution by confocal microscopy confirmed that CD28 ligation did indeed induce the cellular redistribution of SHIP. Under resting conditions, immunofluorescent staining of cells, with anti-SHIP polyclonal anti-serum, revealed a punctate distribution of SHIP protein throughout the cytoplasm (*B* figure 13). However there was a marked re-distribution of SHIP upon CD28 ligation, such that very little SHIP protein remained in the cytoplasm, and that SHIP protein in CD28 stimulated cells was predominantly localised at the membrane (*B* figure 13). Control experiments, using pre-immune rabbit antiserum in place of the primary antibody, revealed no significant staining, demonstrating the specificity of the anti- SHIP polyclonal anti-serum and secondary antibody staining (*B* figure 13).

CD28 COUPLING TO PI3K IS NOT REQUIRED FOR SHIP TYROSINE PHOSPHORYLATION.

Previous work by this group has shown that CD28 and SHIP do not co-precipitate (Edmunds et al 1999), however the inablity to detect an association of SHIP with CD28, may suggest that the coupling of these proteins requires the involvement of intermediate molecules. Earlier work also demonstrated that no co-precipitaton could be detected between p85 and PI3K and therefore the p85 subunit of PI3K is unlikely to physically couple SHIP to CD28 (Edmunds et al 1999). An alternative hypothesis is that PI3K may influence the coupling of SHIP to CD28, through the binding of the D3 phosphoinositide lipid products of PI3K to PH domains of adaptor proteins or to the SH2 domain of SHIP (Han et al 1998, Rameh et al 1995). Thus it could be predicted that D-3 phosphoinositides, formed in response to CD28 ligation, could interact with the SH2 domain of SHIP and recruit SHIP to the membrane where it would be tyrosine phosphorylated by CD28 activated PTKs. Alternatively D-3 phosphoinositides might recruit SHIP indirectly by interacting with PH domain containing adaptor proteins

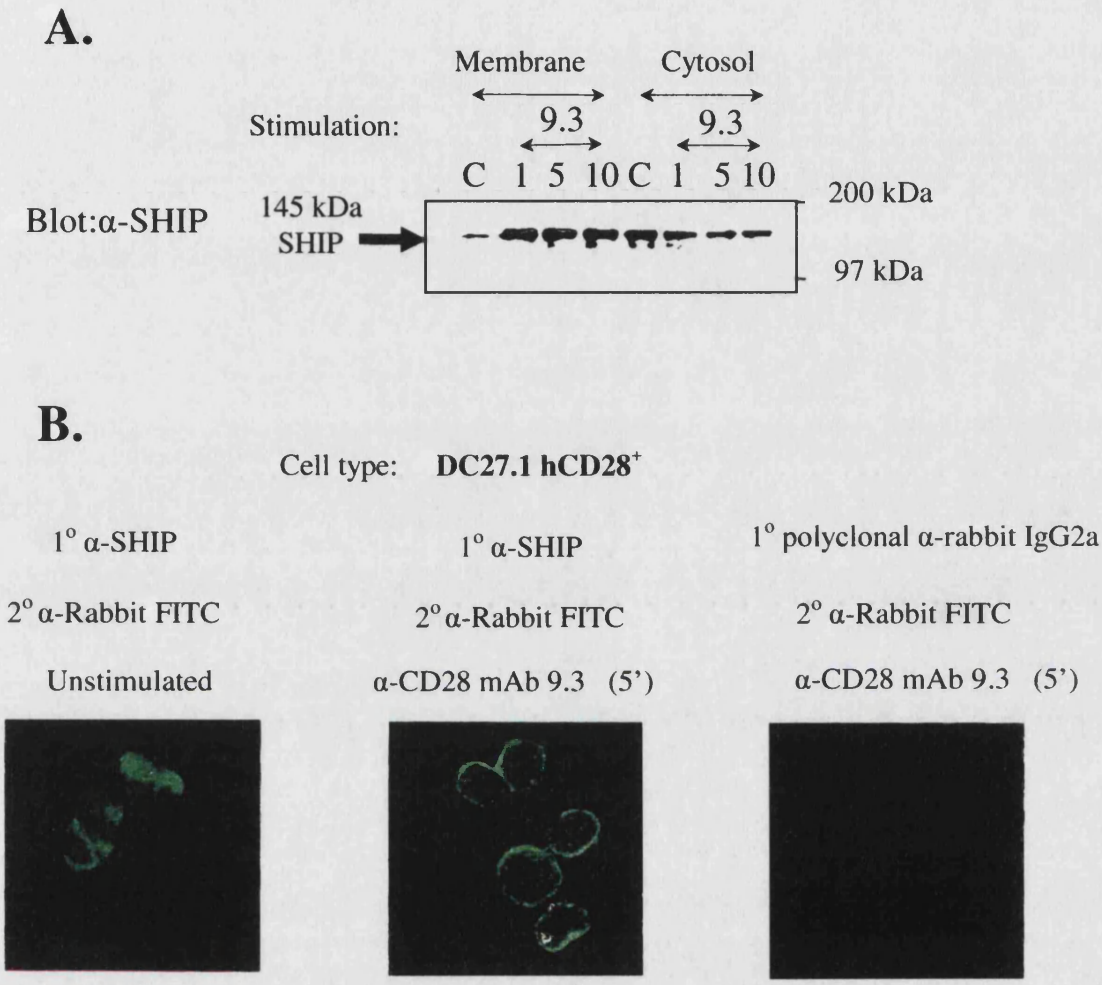


Figure 13: Cellular re-distribution of SHIP in response to α-CD28 ligation

$20x10^6$ cells DC27.1 $CD28^+$ T cell hybridoma cells were stimulated with 5 μg anti CD28 mAb 9.3, or left unstimulated as a control. Cytoplasmic and membrane fraction were purified as detailed in Materials and Methods, and separated by 7.5% SDS-PAGE, prior to transfer onto nitrocellulose for immunoblotting with anti-SHIP Ab (1 μg pre ml in 0.05% Marvel PBS). Proteins were visualised using ECL (Panel A).

$1x10^6$ DC27.1 $CD28^+$ T cell hybridoma cells were either stimulated with 5 μg anti-CD28 antibody 9.3, or left unstimulated as a control, and cytospun at 1,000RPM onto a microscope slide. The slide was then fixed for 10' with 1% paraformaldehyde and permeabilised with acetone and then stained with either, anti-SHIP polyclonal antiserum, or anti rabbit polyclonal anti serum. Slides were washed thoroughly with PBS/Tween and coated with anti-Rabbit FITC antibody. Cells were then visualised by confocal microscopy (Panel B).

and/or protein tyrosine kinases, which may then be responsible for the observed recruitment to the plasma membrane, and/or tyrosine phosphorylation of SHIP.

To investigate the possibility of the PI3K dependency of CD28 mediated tyrosine phosphorylation of SHIP, the ability of CD28 to mediate tyrosine phosphorylation was compared in DC27.1 cells expressing either wild type CD28 or site-specific CD28 mutants in which the PI3K binding motif around $Y1^{173}$ as well as around Y^{200} (which has also been implicated in the recruitment of PI3K (Pages et al 1996)) were mutated to Phe (ΔYF173/200). It has been previously demonstrated using this mutant that disruption of Y^{173} and Y^{200} prevents CD28 coupling to PI3K and D-3 phosphoinositide accumulation following CD28 ligation (Pages et al 1996). However use of the YF173/200 mutant revealed that such mutagenesis of CD28 had little effect on CD28 stimulated tyrosine phosphorylation of SHIP compared to that observed in wild type CD28-expressing DC27.1 cells (Top panel, Figure 14). Efficiency of immunoprecipitation was confirmed by stripping an re-probing blots with anti-SHIP polyclonal antiserum (Bottom panel, Figure 14).

SUMMARY

1. 9.3 mAb ligation of CD28 results in the tyrosine phosphorylation of the 145 kDa SHIP in the DC27.1 T cell hybridoma.
2. FCγRIIB stimulation does not contribute to SHIP tyrosine phosphorylation by 9.3 mAb stimulation.
3. Co-stimulation of CD3 and CD28 receptors stimulates tyrosine phosphorylation of SHIP in an additive fashion.
4. CD28 stimulated tyrosine phosphorylation of SHIP correlates with the redistribution of SHIP from the cytosol, to the plasma membrane.
5. CD28 stimulated tyrosine phosphorylation of SHIP is independent of CD28 coupling to PI3K.

3.2.2 SHIP AND CTLA4

As described in the introduction to this study the CD28 relative, CTLA4, has been described to play a negative regulatory role in T cell activation. The signal transduction pathways by which CTLA4 achieves this role are not yet fully understood. CTLA4 is

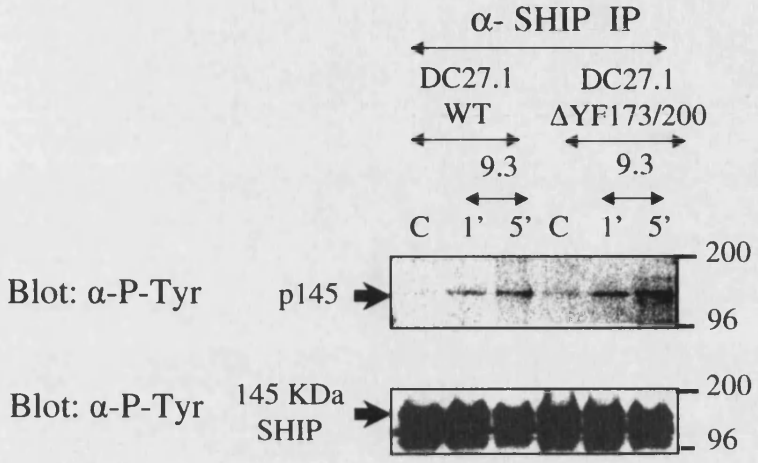


Figure 14: Tyrosine phosphorylation of 145 KDa SHIP following CD28 ligation, in DC27.1 wild type CD28$^+$ and the mutant CD28$^+$ T cell hybridoma.

DC27.1 hCD28 transfected, and DC27.1 hCD28 ΔYF173/200, T cell hybridoma cells were either left unstimulated as a control or stimulated with 5 μg anti hCD28 antibody, 9.3. $20x10^6$ cells were lysed and proteins immunoprecipitated using 1μg anti-SHIP polyclonal antiserum. The resultant proteins were separated by SDS-PAGE and transferred onto nitro-cellulose for immunoblotting with anti-phosphotyrosine antibody, 4G10 (1μg per ml in 0.05% Marvel). Blots were subsequently stripped and re-probed with anti-SHIP polyclonal antiserum at (1μg per ml in 0.05% Marvel) to verify equal loading of immunoprecipitated proteins. These data are from a single experiment and are representative of three separate experiments.

transiently expressed on the T cell surface 2-3 days after T cell activation. It has been demonstrated to associate with PI3K via a YXXM motif in its cytoplasmic tail. In an attempt to elucidate the nature of these signalling cascades this study describes the effects of CTLA4 triggering, by a panel of antibodies, on the production of the PI3K metabolic product PI(3,4,5)P_3, and also describes a role for SHIP in mediating the CTLA4 signal. As CTLA4 is known to play a negative regulatory role in T cell function, and CTLA4 ligation had been demonstrated by data presented below to result in a decrease in cellular PIP(3,4,5)P_3 levels and a concomitant rise in PI(3,4)P_2, it seemed plausible that the lipid 5'-phosphatase SHIP might be regulated by CTLA4 triggered signalling cascades.

INTRACELLULAR AND SURFACE STAINING OF CTLA4.

The study of CTLA4 signalling pathways is hindered by the fact that surface expression of this protein is negligible due to constitutive internalisation via AP-1 mediated endocytosis (Bradshaw et al 1997). Comparison of CTLA4 surface versus intracellular expression demonstrated by the DC27.1 CTLA4$^+$ cell line (kind gift from Chris Rudd) using the anti CTLA4 mAb 11D4, identified that although the majority of protein was internalised, moderate CTLA4 surface expression was apparent in this cell line (Figure 15).

PROLIFERATION IN RESPONSE TO ANTI CTLA4 MABs.

To characterise the *in vitro* effects of CTLA4 ligation, induced by CTLA4 antibodies, 3D6 and CT29 (generously provided by Carl June), the ability of these antibodies to impair CD3/CD28 induced proliferation of freshly purified primary T cells was measured. CTLA4 mAbs had been previously coupled Dynal^{TM} beads, at a ratio of 8:1:1 with anti CD3 and anti CD28 abs respectively.

In vitro proliferative responses, measured via ^{3}H incorporation, show that 3D6 and CT29 anti CTLA4 antibodies are able to inhibit CD3 and CD28 mAb stimulated proliferation (Figure 16). It appeared that these antibodies were able to mimic the proliferative effects of CTLA4 stimulation *in vitro* (Krummel et al 1995, Walanus et al 1996) that had previously been described, and that they were suitable for further molecular characterisation of the CTLA4 mediated inhibitory signal.

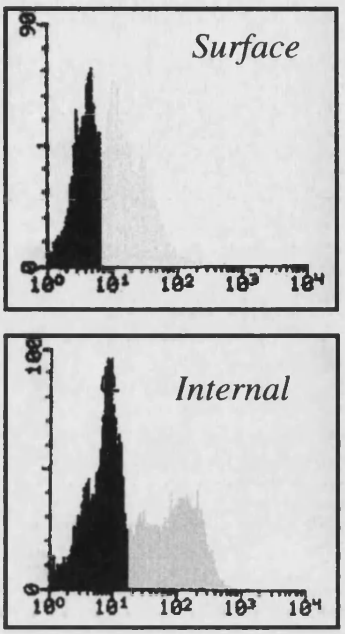


Figure 15: Intracellular and surface FACS staining for CTLA4 expression

$5x10^5$ CTLA4 expressing hybridoma cells were either fixed with paraformaldehyde and permeabilised with saponin, for intracellular staining, or left unfixed for surface staining, and incubated for 1 hr at 4 ^{o}C with 1 μgml^{-1} either anti-CTLA4 antibody, 11D4 (grey histogram), or an isotype matched murine IgG2a as a negative control (black histogram). After washing cells were incubated with anti-mouse FITC for 30 minutes at 4 ^{o}C, then washed and analysed for CTLA4 expression by flourescence activated cell sorting (FACS).

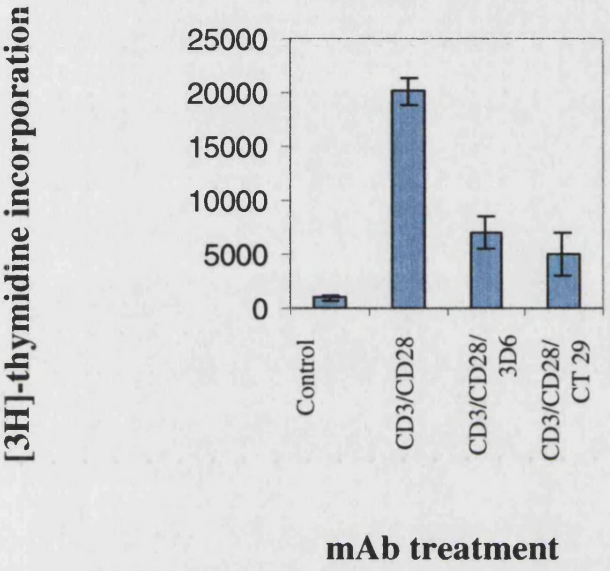


Figure 16: Proliferation of primary T cells in response to CTLA4 CD3 and CD28 stimulation.

Freshly purified T cells were incubated with anti-CD3 anti-CD28 and either anti CTLA4 antibody 3D6, or anti CTLA4 antibodyCT29. Plates were pulsed at 48hrs with [^{3}H] thymidine, and harvested 24 hrs later. Proliferation was measured via the incorporation of [^{3}H] thymidine, in response to each set of stimuli. These data represent the mean ± SEM from three separate experiments.

ACCUMULATION OF D3 INOSITOL LIPIDS IN RESPONSE TO CTLA4 STIMULATION.

Having verified the functional consequences of CTLA4 ligation via anti CTLA4 mAbs, CT29 and 3D6, their effects on biochemical readouts were ascertained. Using the CTLA4 expressing murine T cell hybridoma line, cells were stimulated with anti CTLA4 mAb 3D6 and CT29. $PI(3,4,5)P_3$ levels measured in resting cells demonstrated moderate levels of basal PI3K activity (Figure 17), which, upon ligation with either 3D6 or CT29 decreased considerably (Figure 17). Conversely measuring cellular levels of $PI(3,4)P_2$ demonstrated a clear increase in levels of this phosphoinositide following CT29 and 3D6 treatment, suggesting that ligation of CTLA4 promotes the dephosphorylation of $PI(3,4,5)P_3$ resulting in a steady increase in $PI(3,4)P_2$.

ACTIVATION OF SHIP IN RESPONSE TO STIMULATION THROUGH CTLA4

To investigate whether SHIP is implicated in signalling pathways initiated by CTLA4, the $CTLA4^+$ T cell hybridoma was used. CTLA4 was either ligated by its natural ligand B7.1, or by anti-CTLA4 antibodies 3D6 and CT29, whilst anti mouse CD3 mAb, 2C11, was used to stimulate cells as a positive control and anti-human CD28 and anti hCD3 mAb (UCHT1) were used as negative controls. It must be noted that B7.1 can stimulate mCTLA4 and mCD28 in addition to the hCTLA4 receptor expressed by this cell line. Stimulated and resting cells were lysed, immunoprecipitated with anti-SHIP polyclonal antiserum, and immunoblotted with anti-phosphotyrosine mAb 4G10. The presence of a 145-150kDa band, which increased in intensity upon stimulation with B7.1, 3D6 and was particularly prominent following stimulation with CT29, suggested that SHIP activation might be a downstream effect of CTLA4 stimulation (figure 18). Similarly, stimulation through mCD3 with 2C11 mAb produced tyrosine phosphorylation of this protein (Figure 18). Finally stimulation with anti hCD28 mAb 9.3, and anti hCD3 mAb UCHT1 for 5 minutes did not induce the tyrosine phosphorylation of 145 kDa SHIP. Immunoblotting with anti-SHIP polyclonal antiserum confirmed the identity of the 145 kDa phospho-protein as SHIP, and demonstrated equal loading, and transfer, of immunoprecipitated proteins (Figure 18).

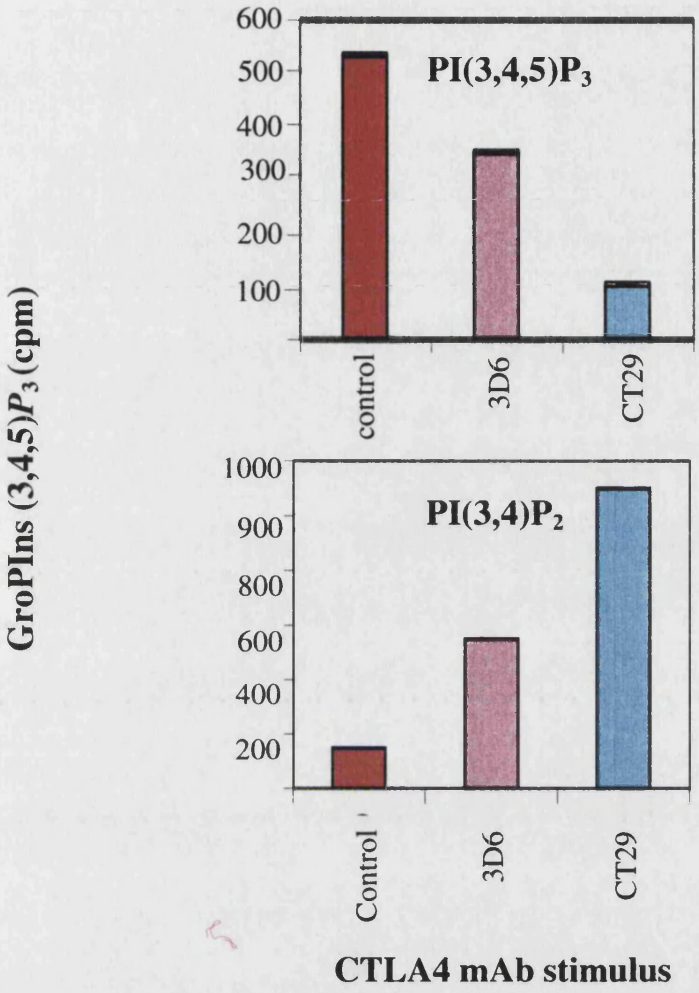


Figure 17: D-3 phosphoinositide lipids accumulation of response to anti-CTLA4 stimulation.

$20x10^6$ DC $CTLA4^+$ T hybridoma cells were labelled per point with [^{32}P]Pi for 3 hrs at 37°C in phosphate free medium supplemented with 10% dialysed FCS and HEPES. Cells were then stimulated with anti-CTLA4 antibodies, 3D6 and CT29 for 5 minutes. Reactions were quenched with chloro-form:methanol, and the phosohatidylinositol lipids were extracted from each sample as described in materials and methods. Finally samples were de-acylated prior to analysis via HPLC. Results are plotted as actual peak area. **Actual counts** = $PI(3,4,5)P_3$: Control: 587; 3D6: 348; CT29:123cpm; $PI(3,4)P_2$: Control: 150; 3D6: 550, CT29: 1110 cpm. **Total counts** = 1,150,00cpm.

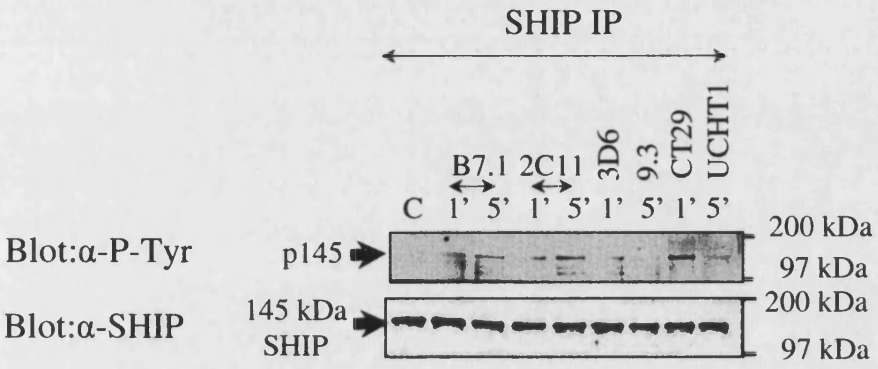


Figure 18: Tyrosine phosphorylation of SHIP in response to CTLA4 ligation.

$2x10^7$ CTLA4 expressing T cell hybridoma cells, DC 27.1, were either left unstimulated as a control (C), or stimulated with either CHO-B7.1$^+$ cells (at a ratio of 3:1 respectively), 5 μg anti-CTLA4 mAbs, 3D6 and CT29, anti-mCD3 mAb, 2C11 or, as negative controls, 5 μg anti-hCD28 antibody 9.3, or anti-hCD3 antibody UCHT1. Cells were lysed and subject to immunoprecipitation with 1 μg anti- SHIP polyclonal anti-serum. Precipitated proteins were separated by 7.5 % SDS-PAGE and transferred onto nitrocellulose membranes for immunoblotting with anti-phosphotyrosine antibody, 4G10 at 1 μg per ml in 0.05 % marvel/PBS. Blots were stripped and re-probed with anti-SHIP polyclonal anti-serumat 1 μg per ml in 0.05 % to verify equal loading of immunoprecipitated proteins. These data are from a single experiment which is representtive of two other experiments.

TYROSINE PHOSPHORYLATION OF SHIP IS A SPECIFIC CTLA4 MEDIATED EVENT

As described earlier (section SHIP 3.2) and by Chacko et al (1996), FCγRIIB ligation strongly induces the tyrosine phosphorylation of SHIP in B lymphocytes. To rule out the possibility that FCγRIIB receptor binding, by FC regions of the anti- CTLA4 mAbs used in this study, can contribute to the SHIP tyrosine phosphorylation observed following CTLA4 stimulation, a F(ab')2 fragment of anti CTLA4 mAb, CT29 was utilised. F(ab')2 fragments of antibodies lack FC regions and so would not be able to bind non specifically to FCγRIIB receptors.

$CTLA4^+$ hybridoma T cells were stimulated with anti-CTLA4 CT29 (F(ab')2 and immunoprecipitated with anti-SHIP polyclonal antiserum. Immunoblotting with anti phosphotyrosine antibody 4G10 revealed that a 145-150 kDa band was heavily tyrosine phosphorylated in response to CTLA4 ligation by CT29 F(ab')2 (Figure 19). Immunoblotting with anti-SHIP polyclonal antiserum identified this protein as SHIP and verified equal loading of immunoprecipitated protein (Figure 19). Therefore tyrosine phosphorylation of SHIP following treatment with anti-CTLA4 mAbs is not attributable to FC receptor triggered PTK activity and must therefore be a direct consequence of CTLA4 ligation.

SUMMARY

1) Anti-CTLA4 mAbs, CT29 and 3D6 inhibit CD28 / CD3 mediated proliferation of primary T cells *in vitro*.
2) Stimulation of the CTLA4 expressing T cell hybridoma DC27.1via anti-CTLA4 mAbs, CT29 and 3D6 mediates the reduction of cellular PI(3,4,5)P_3 levels, which correlates with an increase in levels of PI(3,4)P_2.
3) Stimulation of DC27.1 with either anti-CTLA4 mAbs, CT29 and 3D6, anti-murine CD3 mAb, 2C11 or CHO-$B7.1^+$ cells induces rapid tyrosine phosphorylation of the inositol 5'-polyphosphatase SHIP.

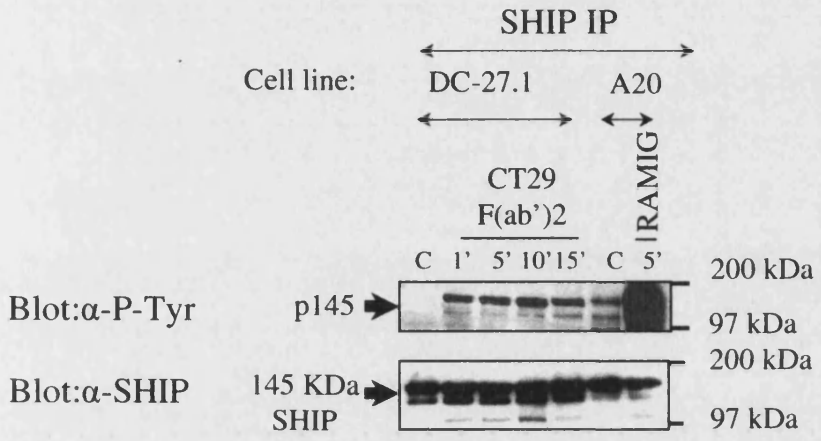


Figure 19: F(ab')2 CT29 induced tyrosine phosphorylation of SHIP in the CTLA-4 expressing T cell hybridoma DC.

DC27.1 Cells were either left un-stimulated as a control or stimulated using 5 μg anti- CTLA-4 F(ab') CT29. $20x10^6$ cells per point were lysed and immunoprecipitated with 1 μg anti- SHIP. Precipitates were separated by 7.5 % SDS-PAGE and transferred to nitro-cellulose for immunoblotting with anti-phosphotyrosine antibody 4G10 at 1 μg per ml in 0.05% Marvel in PBS. Blots were stripped and reprobed with anti- SHIP to verify equal loading of immuno-precipitated proteins. Proteins were visualised via chemiluminescence.

3.2.3 REGULATION OF BASAL LEVELS OF 3'PHOSPHOINOSITIDES

This study has described the identification of 145 kDa SHIP as a target for CTLA4 and CD28 mediated signals in T lymphocytes. SHIP's role in the regulation of PI3K dependent signalling cascades in T lymphocytes, via the enzymatic degradation of the phosphoinositide lipid product PI(3,4,5)P_3, has therefore been discussed. The lack of detectable levels of SHIP expression in the Jurkat T cell line has been previously demonstrated, and recently the lack of expression of the tumour suppressor PTEN has also been reported (Shan et al 2000). As such, the study of the Jurkat T cell line as a SHIP and PTEN 'null model' may provide useful insight into the role of inositol polyphosphatases in the regulation of PI(3,4,5)P_3 levels in T cells. We sought to investigate the effect of SHIP and PTEN deficiency on constitutive levels of D-3 lipids and PKB activation in J6 versus A20 cells.

3'-PHOSPHOINOSITIDE IN RESTING JURKAT T CELLS VERSUS A20 B CELLS.

Firstly, it was considered whether the absence of SHIP expression in Jurkats influences basal levels of 3-phosphoinositides in this cell line. Metabolic labelling experiments, in which phosphoinositide lipids were labelled with [^{32}P]- orthophosphate, were carried out and 3-phosphoinositide levels in Jurkat T cells were compared to those in unstimulated A20 BALB/c mouse B lymphoma cells. It was observed (table 5) that unstimulated Jurkats exhibit basal levels of PI(3,4,5)P_3 levels which are approximately five fold greater than basal levels of PI(3,4)P_2. Upon ligation of the CD28 receptor complex (TCR), there was a two fold increase in the PI(3,4,5)P_3 levels above basal. In contrast, in the A20 cell line, basal levels of PI(3,4,5)P_3 were low and basal levels of the 3'-phosphoinositide PI(3,4)P_2 were more prominent. Triggering of the B cell antigen receptor (BCR) induced a 10-12 fold increase in detectable levels of PI(3,4,5)P_3 (table 5) and just over a two fold increase in PI(3,4)P_2.

DETECTION OF SHIP IN VARIOUS T CELL LINES

In order to demonstrate the absence of SHIP expression in Jurkat T cells in comparison to primary T lymphoblasts and A20 B cells, whole cell lysates were prepared from each of these cell types and blotted with anti-SHIP polyclonal antiserum.

Treatment	PI(3,4)P_2 (cpm)	PI(3,4,5)P_3 (cpm)
A20 Cells:		
Basal	2008 ± 381	777 ± 293
F(ab')$_2$	5499 ± 665	9863 ± 2345
Jurkat Cells:		
Basal	565 ± 335	3431 ± 1021
9.3	836 ± 350	6373 ± 1638

Table 5: Basal and stimulated levels of 3'-phosphoinositide lipids in Jurkat and A20 Cells.

$2x10^7$ [^{32}P]-labeled Jurkat cells or A20 cells were left unstimulated or stimulated at 37 °C with 10 mg/ml anti-CD28 mAb 9.3 for 10 minutes or with 12μg F(ab')$_2$ fragments of rabbit anti-mouse IgG (RAMIG) for 5 minutes respectively. The 3'-phosphoinositide lipids were extracted, deacylated and the glycerophosphorylinositol derivatives of PI(3,4)P_2 and PI(3,4,5)P_3 analysed by HPLC separation. Data is mean cpm values ± standard errors from at least five experiments.

This demonstrated that, in accordance with earlier data by this group and others, SHIP expression cannot be detected in Jurkat T cells (figure 20), whilst it was readily detectable in whole cell lysate derived from A20 B cells and T lymphoblasts (figure 20). (NB: only a single SHIP isoform could be detected, which represented 145 kDa SHIP. This was most likely due to the use of a different anti-SHIP antibody for this experiment, than had been used in the prior sections of this study).

DETECTION OF PTEN PROTEIN IN A20 CELLS AND CTLA4 EXPRESSING HYBRIDOMA CELL LINE DC27.1.

Previous work has shown that PTEN is not expressed in J6 (Shan et al 2000). To investigate whether PTEN was expressed in the cell models used in this study whole cell lysates derived from the A20 B cell line and the DC27.1 hybridoma cell line were immunoblotted with anti PTEN mAbs. PTEN is a more recently described lipid phosphatase which dephosphorylates $PI(3,4,5)P_3$ at the D-3 position and could thus contribute to the abrogation of accumulation of D-3 phosphoinositides observed in response to FCγRIIB co-ligation. It was therefore considered whether PTEN could be a potential regulator of PI3K lipid products in A20 cells, and moreover in lipid signalling pathways in T cells.

To establish whether A20 cells express the lipid phosphatase PTEN cell lysates were titrated from $20x10^6$ to $2x10^6$ cell equivalents (figure 21). In addition lysates of the T cell hybridoma, DC27.1, were titrated in a similar manner. Immunoblotting with anti-PTEN antibody revealed a band of approximately 50 kD which was visible down to $2x10^6$ cell equivalents in the DC27.1 cell line but was only faintly visible at 20 and $10x10^6$ cell equivalents in A20 cells (Figure 21).

CELLULAR LOCALISATION OF GFP-PKB-PH IN JURKAT T CELLS

Having identified basally high levels of 3'-phosphoinositides exhibited by Jurkat T cells, a construct expressing the green fluorescent protein-tagged pleckstrin homology domain of the serine threonine kinase, protein kinase B (PKB), GFP- PKB PH (kindly provided by Julian Downward ICRF, London) was transfected into Jurkats. The binding of phosphoinositides to the PH domain of this construct determines the membrane localisation of GFP in cells. The GFP-PH-PKB construct has been used previously in B cells (Astoul et al 1999) to visualise the membrane re-localisation of PKB upon

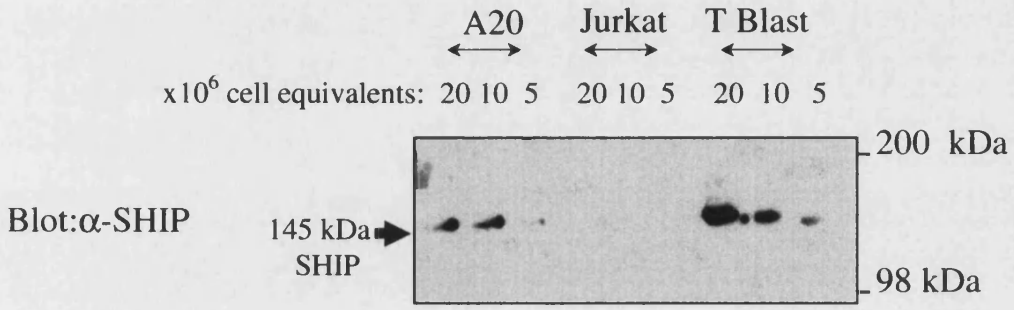


Figure 20: Expression of 145 kDa SHIP in A20 B cells, Jurkat T cells, and cultured T lymphoblasts.

$2x10^6$ A20 B cells, Jurkat T cells, and cultured T lymphoblasts were lysed and acetone precipitated woth 0.7 volumes of ice cold acetone. Following centrifugation, proteins were separated via 7.5 % SDS PAGE and transferred onto nitro-cellulose for immunoblotting with anti-SHIP polyclonal antiserum (Santa Cruz) at a concentration of 1 μg per ml in 0.05 % marvel/PBS. These data are from a single experiment and are representative of three other experiments.

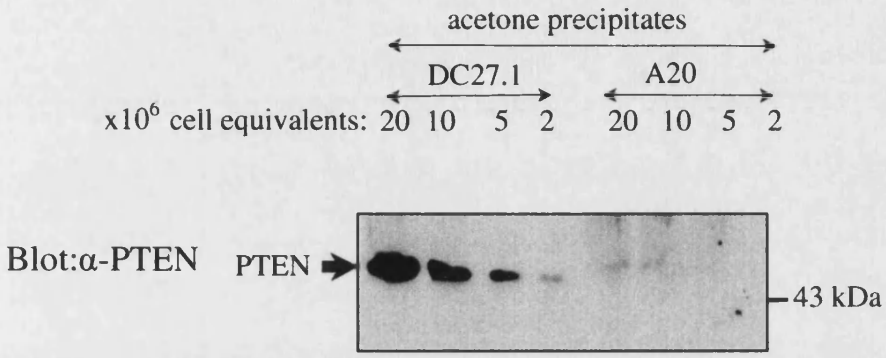


Figure 21: Differential expression of PTEN in CTLA4 hybridoma and A20 cells.

DC27.1 CTLA4$^+$ and A20 cells were lysed at the cell densities shown. Proteins were acetone precipitated and separated via 7.5 % SDS-PAGE, transferred onto nitro-cellulose, and immunoblotted with anti-PTEN mAb at a concentration of 1μg per ml in 0.05 % marvel/PBS. Proteins were visualised by chemiluminescence. These data are from a single experiment ahich was representative of two others.

antigen receptor ligation: In resting A20 cells, GFP-PH-PKB was cytoplasmically localised and BCR ligation triggered re-localisation to the membrane. This correlated with the phosphorylation of endogenous PKB. Overexpression of active PI3K (CD2-p110) by this study (Astoul et al 1999) elicited the constitutive membrane recruitment of GFP-PH-PKB.

Localisation of GFP-PH-PKB at the membrane in Jurkat T cells (figure 22, *top left* panel) correlated with the basally high levels of 3'-phosphoinositides measured in this cell line. As a negative control transfection of Jurkats with a non 3' phosphoinositide binding PKB-PH domain mutant (GFP- PKB-PH-$R^{25}C$). (Rameh et al 1997), and immunofluorescent staining, demonstrated the predominant localisation of GFP throughout the cytosol in a diffuse manner, with non fluorescing areas indicating exclusion of the GFP from the nucleus (figure 22 *top right* panel).

LY249002 INHIBITION OF PI-3K INDUCES CELLULAR REDISTRIBUTION OF GFP-PKB-PH

To gauge the dependency of membrane localisation of GFP-PKB-PH on D-3 lipids, the PI3K inhibitor LY249002 was used. The re-localisation of transfected GFP-PKB-PH was visualised via immuno-fluorescent-microscopy. GFP-PH-PKB was predominantly detected at the membrane until 30 minutes post LY249002 treatment (figure 23), after which a significant re-localisation to the cytoplasm could be observed. At 1 hour post LY94002 treatment, the majority of GFP-PKB-PH could be visualised in the cytosol (figure 23).

CHARACTERISATION AND EXPRESSION OF CD2-SHIP CONSTRUCTS

To investigate the effects of overexpressing catalytically active and mutant SHIP against a null SHIP background, Jurkat T cell were transiently transfected with rCD2-SHIP and rCD2-SHIP-$C^{671}A$ which were generously provided by Doreen Cantrell, ICRF London. These constructs comprised the minimum catalytic core (aa 364-825) of SHIP, or $C^{671}A$ mutants of this sequence fused to the intra-cytoplasmic domain of CD2, thus localising active or dominant negative to the plasma membrane (figure 24 A.).

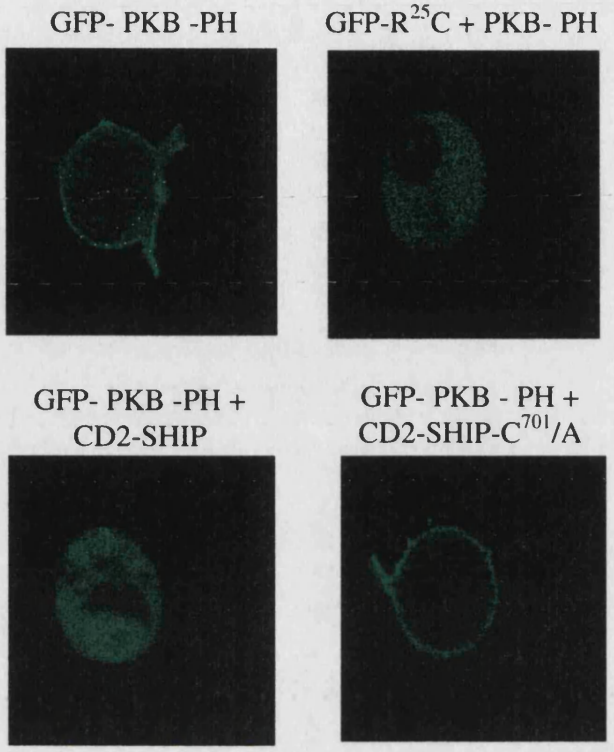


Figure 22: Cellular re-localisation of GFP-PH-PKB by co-expression of rCD2-SHIP and rCD2-SHIP-C^{701}/A.

Jurkat T cells were transiently transfected at $1.5x10^7$ cells per ml by electroporation with green fluorescent protein tagged PKB-pleckstrin homology domain cDNA, GFP-PH-PKB, or a non-lipid binding PH mutant GFP-R^{25}C PH-PKB, using 10 µg of plasmid per $1.5x10^7$ cells. In addition cells were transiently co-transfected with 30 µg of cDNA representing the catalytic domain of SHIP fused to the CD2 transmembrane domain, CD2-SHIP, or a catalytically dead SHIP enzymatic domain fused to the CD2 transmembrane domain CD2-SHIP-C^{701}/A. Following electroporation cells were re-suspended in RPMI, 10% FCS and layered onto cover slips in 24 well plates. Cells were incubated at 37°C for five hours, and then washed in PBS and fixed for 10' with 1% paraformaldehyde and then visualised by confocal microscopy.

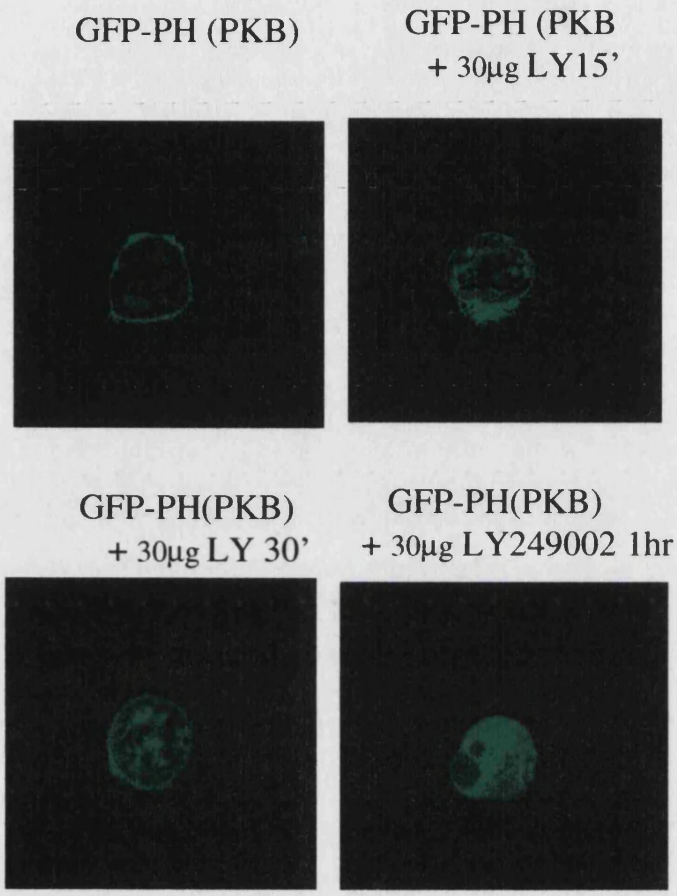


Diagram 23: Inhibition of GFP-PH (PKB) membrane localisation by Ly249002

Jurkat T cells were transiently transfected at $1.5x10^7$ cells per ml by electroporation with green fluorescent protein tagged PKB-pleckstrin homology domain cDNA, GFP-PH-PKB, using 10 μg of plasmid per $1.5x10^7$ cells. Following electroporation cells were re-suspended in RPMI, 10% FCS and layered onto cover slips in 24 well plates. Cells were treated at the times indicated with 30μM LY249002 prior to fixing with 1% paraformaldehyde and visualisation via confocal microscopy.

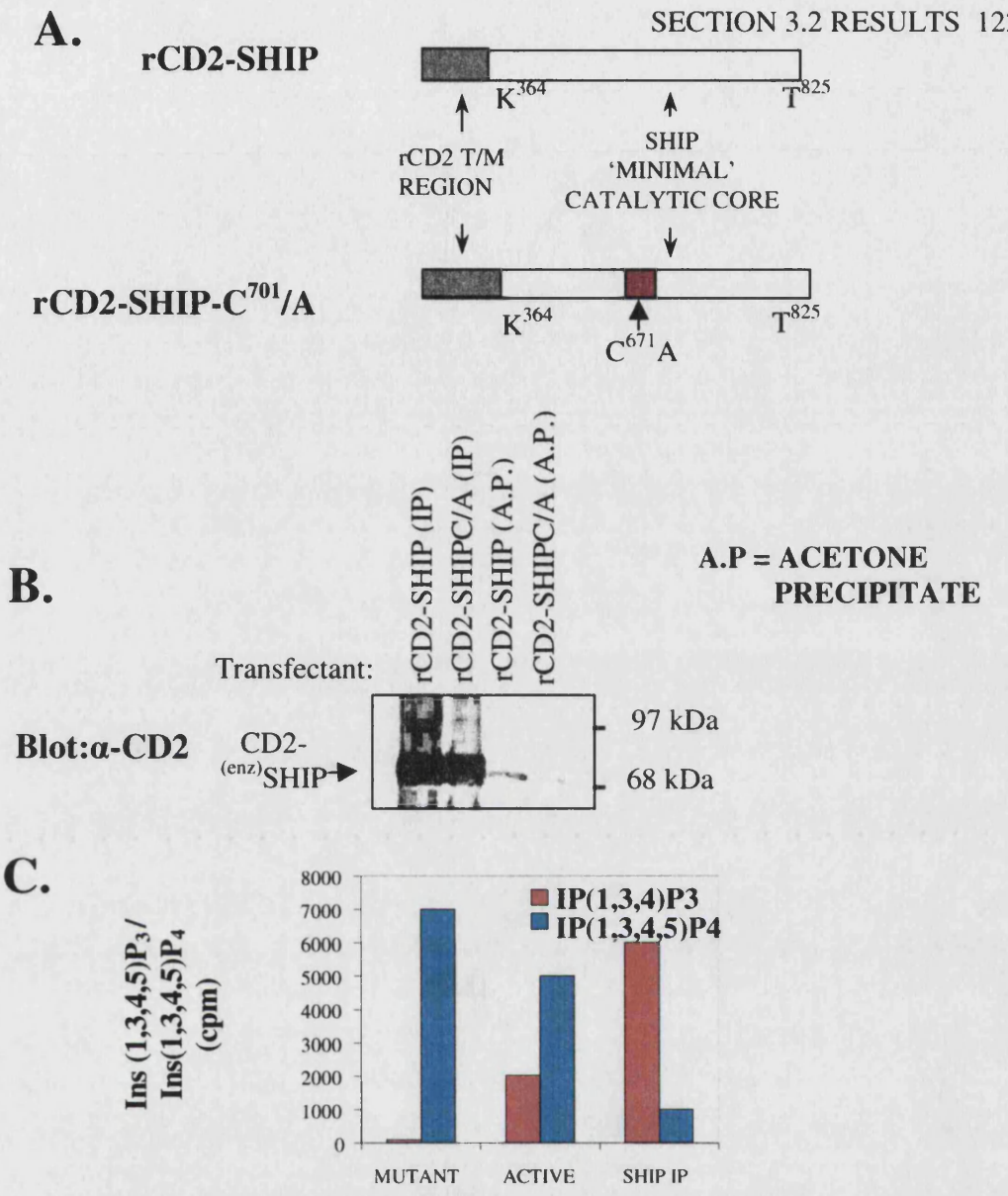


Figure 24: Analysis of 5'-polyphosphatase activity of CD2-(enz)SHIP and CD2-(enz)ΔSHIP constructs.

$15x10^6$ Jurkat T cells per ml were electroporated with 30 µg rCD2-SHIP or rCD2-SHIP-C^{671}A (**A.**). Following electroporation cells were resuspended in RPMI 10% FCS, and allowed to express overnight. $2x10^7$ cells were lysed and immunoprecipitated with 1 µg anti-CD2 antibody OX34. As a control $2x10^7$ DC27.1 cells were lysed and 100 µl of lysate was removed, and acetone precipitated as a control and precipitated with 1 µg anti-SHIP polyclonal anti-serum Precipitated proteins were equally split and either separated via 7.5% SDS/PAGE and immunoblottted with anti CD2 mAb Ox34 at a concentration of 1 µg per ml in 0.05 % Marvel/PBS (**B.**); alternatively IPs assayed for 5' phosphatase activity by measurement of *in vitro* hydrolysis of [^{3}H]-ins(1,3,4,5)P_4 to [^{3}H]-ins(1,3,4)P_3. Data are from a single experiment (**C.**).

To determine the *in vitro* phosphatase activity of rCD2-SHIP and rCD2-SHIP-$C^{671}A$, anti-rCD2 immunoprecipitates, derived from Jurkat cells which had been transfected with these constructs, were assayed for their ability to catalyse the degradation of [^{3}H]-Ins (1,3,4,5)P_4 to [^{3}H]-Ins (1,3,4)P_3 (Figure 24 C.). The rCD2-SHIP construct catalysed the degradation of 98.9 % [^{3}H]-Ins (1,3,4,5)P_4. Whilst the anti-CD2 immunoprecipitates from the rCD2-SHIP-$C^{671}A$ transfected cells were able to catalyse the degradation of 28 % of the [^{3}H]-Ins (1,3,4,5)P_4. Meanwhile the anti SHIP immunoprecipitate from the resting DC27.1 cell lysate catalysed the degradation of 90% of the [^{3}H]-Ins (1,3,4,5)P_4. Comparative expression of constructs was established by immunoblotting samples of acetone precipitated lysates and anti-rCD2 immunoprecipitated protein from transfected samples, with anti-CD2 mAbs (figure 24B.).

CELLULAR RE-LOCALISATION OF GFP-PKB-PH IN JURKAT T CELLS VIA THE CO-EXPRESSION OF CD2-SHIP

The *in vitro* assays proved to be inconsistent thus an alternative method was therefore sought by which to assess the activity of rat CD2-SHIP constructs. Hence, given that this study had previously shown that J6 do not express either PTEN or SHIP and that they express elevated levels of PI(3,4,5)P_3, (which correlate with constitutive membrane localisation of GFP-PKB-PH domains) it was reasoned that co-expression of rCD2-SHIP mutants with GFP-PKB-PH would assess the effect of rCD2-SHIP constructs on the localisation of the GFP- tagged PH domains.

Cells were co-transfected with GFP- PKB PH and either rCD2-SHIP or rCD2-SHIP-$C^{671}A$ and subject to immunofluorescence microscopy. As a negative control transfection of Jurkats with PKB-PH domain mutant (GFP- PKB-PH-$R^{25}C$) (Rameh et al JBC 1997) and immunoflourescent staining demonstrated the predominant localisation of GFP in these cells throughout the cytosol in a diffuse manner, with non fluorescing areas indicating exclusion of the GFP from the nucleus.

Confocal imaging of GFP-PKB-PH and CD2-SHIP co-transfected cells revealed a marked re-distribution of the GFP-PKB-PH protein from the plasma membrane to the cytoplasm of the cell (figure 22, *bottom left hand panel*), when compared with cells transfected with GFP-PKB-PH alone. The diffuse localisation of fluorescence across the

cytoplasm outlined the lobular nucleus as a non fluorescing area, indicating the exclusion of GFP protein from this structure. Meanwhile co-expression of CD2-SHIP with GFP-PKB-PH, had no effect on the distribution of GFP-PKB-PH, which retained a strict plasma membrane co-localisation (figure 22, *bottom right hand panel*).

SUMMARY

1) Jurkat T cells exhibited high basal levels of PI(3,4,5)P_3 and PI(3,4)P_2. These basal levels of 3'-phosphoinositide are demonstrated here to mediate the retention of GFP-PKB-PH at the plasma membrane.

2) Inhibition of constitutive PI3K activity via LY249002 treatment of Jurkat cells, mediated the re-localisation of GFP-PKB-PH from the plasma membrane into the cytosol which occurred after 30 minutes and was complete at 1 hour.

3) Overexpression of rCD2-SHIP in Jurkats, which has been demonstrated here to convey *in vitro* 5' phosphatase activity on [^{3}H]- Ins (1,3,4,5)P_4, mediates a marked cytosolic re-localisation of GFP-PKB-PH away from the membrane.

3.2 DISCUSSION

3.2.1 CD28 AND SHIP

145 kDa SHIP is classified as a type III member of the family of inositol-5'-poly phopshatases which are outlined in the introduction to this study. SHIP specifically degrades the D-3 phosphoinositide PI(3,4,5)P_3 to PI(3,4)P_2 and has been heavily implicated by this study in the signalling cascades regulated by the TCR/CD3 complex and CD28. Data provided by this study indicate that SHIP is a biochemical target for CD28 mediated signals and may play a role in regulating T cell activation in response to costimulatory signals. Interestingly, 145 kDa SHIP is not expressed in Jurkat T cells and thus this study employed the use of the $CD28^+$ T-cell hybridoma, DC27.1, which has been previously demonstrated by this group to express high levels of the 145 kDa isoform of SHIP, Previous reports in other cell lines have described an array of SHIP related proteins, and in the DC27.1 cell line additional SHIP related proteins of 155, 135 and 130 were reported to be immunoprecipitated with the 145 kDa isoform by the SHIP polyclonal antiserum used in this study (Edmunds et al 1999). Furthermore the existence of SHIP species of varying molecular weight could account for the multiple SHIP proteins seen by this study when re-probing anti-SHIP immunoprecipitates with anti-SHIP antibody.

Although the 145 kDa isoform is generally the largest form of SHIP consistently detected, additional 160 and 150 kDa SHIP related proteins have also been described (Ono et al 1996, Lioubin et al 1996), and these could represent the 155 kDa SHIP related band seen in whole cell lysates from the DC27.1 hybridoma (Edmunds et al 1999). Additional reports suggest that this band might be SHIP2, which could be putatively detected via antibody cross reactivity. A further 135 kDa band in DC27.1 is likely to be either the 135 kDa splice product, which lacks a 183 nucleotide region downstream of the first NPXPY motif that may alter the specificity of SH2 domains binding this motif. Alternatively this band could represent a 135 kDa catalytic degradation product of the 145 kDa protein which has also been reported (Lucas and Rohrschneider et al 1999).

Detection of these proteins may be cell type specific, as demonstrated here by the differential expression of SHIP proteins observed between Jurkat and T lymphoblast cells. Further, the varying estimates of molecular weight of these bands and their differential recognition by a variety of SHIP specific antibodies has added to the

heterogeneity of SHIP proteins that have been detected, and this has been demonstrated here by the differential detection of SHIP isoforms by antibodies used in this study. An interesting observation, that SHIP proteins of increasing molecular weight can be detected in bone marrow and immature haematopoietic cell lines as differentiation proceeds, suggests that the existence of a variety of SHIP proteins may be of functional significance (Geier et al 1997). It would appear that some of these isoforms are attributable to the specific proteolytic degradation of full length SHIP, which occurs progressively from the C-terminus and may be mediated by a member of the calpain family (Damen et al 1998). Furthermore the existence of the SHIP related SIP-110 (Lucas et al 1999), which lacks the SH2 domain of SHIP and is not phosphorylated in response to growth factor receptor signalling, is derived from alternatively spliced full length SHIP and demonstrates that different SHIP proteins may be regulated by distinct mechanisms.

This study demonstrates that ligation of CD28 via anti CD28 mAbs induces the tyrosine phosphorylation of SHIP. Interestingly, SHIP displays low basal tyrosine phosphorylation in the DC27.1 cell line, which contrasts with reports of high basal SHIP tyrosine phosphorylation in IL-3 dependent (Damen et al 1996) and IL-2 dependent (Lamkin et al 1997) cell lines, whilst reflecting observations made in other T cell lines (Lamkin et al 1997). CD28 ligation via B7.1, the CD28 physiological ligand, stimulates tyrosine phosphorylation of SHIP in a manner consistent with that produced via anti-CD28mAb ligation. Whilst supporting the physiological relevance of CD28 mediated phosphorylation of SHIP, this finding clearly demonstrates the CD28 specificity of this response. Furthermore, it is shown that FCγRIIB/RIIIB ligation via mAb FC regions, which has been described to lead to SHIP phosphorylation in B cells (Chacko et al 1997), has no influence on the tyrosine phosphorylation of SHIP induced in response to CD28 mAbs.

Previous studies have demonstrated that the enhanced phosphorylation of SHIP can be achieved through the simultaneous ligation of the CD3 and CD4 receptors of a T cell hybridoma cell line (Lamkin et al 1997). Similarly, this study has shown that an additive effect on SHIP tyrosine phosphorylation is observed when co-stimulation through the CD3 and CD28 receptors on the DC27.1 hybridoma is administered. It is well established that CD28 can activate PTKs such as $p56^{Lck}$, $p59^{Fyn}$ and Itk (Lu et al 1998, Marengere et al 1997, Ward et al 1996), hence it is possible that one or more

of these kinases may be involved in mediating tyrosine phosphorylation of either intermediate proteins or SHIP in response to CD28 ligation.

Although previous work by this group (Edmunds et al 1999) has shown that the CD28 stimulated increase in 5-phosphatase activity present in SHIP immunoprecipitates correlates with tyrosine phosphorylation of SHIP, a requirement for tyrosine phosphorylation for the enhancement of SHIPs enzymatic activity remains to be demonstrated. Findings described in this study appear to be contradictory to previous observations made which have described that the phosphatase activity of SHIP is negatively regulated by tyrosine phosphorylation, in the context of the IgE receptor mediated signalling pathways (Osborne et al 1996). Furthermore studies have described SHIP immunoprecipitates derived from resting and growth factor stimulated cells (Lioubin et al 1996) display comparative levels of 5- phosphatase activity whilst SHIP's recruitment to Shc or tyrosine phosphorylated complexes following growth factor stimulation is increased. This suggests that neither tyrosine phosphorylation nor coupling to Shc correlate with up-regulation of 5-phosphatase activity (Lioubin at el 1996). Data presented in this study, by previous work (Edmunds et al 1999), therefore indicate that signalling pathways, mediated by CD3 and CD28 in T cells, possess a distinct pattern of events leading to the enzymatic activation of SHIP to those which regulate SHIP in growth factor mediated signalling pathways. At present, however, the function that tyrosine phosphorylation of SHIP plays in the regulation of 5-phosphatase activity is still unclear.

A further possibility exists in that the tyrosine phosphorylation of SHIP could be required for the adaptor protein mediated re-localisation of SHIP to the plasma membrane. The tyrosine residues located within the C terminal NPXPY motif of SHIP have been previously demonstrated to interact with Shc phosphotyrosine binding domain (Lamkin et al 1997) and, more recently, the N-terminal phosphotyrosine binding domain of p62 Dok-1 has been demonstrated to associate with the same SHIP motif (Tamir et al 2000). Furthermore, as the SHIP SH2 domain does not participate in these interactions it may be free to bind additional proteins which might influence its function. Thus SHIPs relocalisation with the plasma membrane through the interaction of its phosphotyrosine residues with the PTB domains of other signalling proteins may mediate its approximation with its lipid substrates in the membrane, and thus up-regulate its enzymatic function. In

accordance with this model, data described by this study have demonstrated the effects of CD28 ligation on the cellular re-distribution of SHIP to the membrane.

SHIP has previously been reported to associate directly with a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM) present in the cytoplasmic domain of FCγRIIB, I/LxYxxL (Tridandapani et al 1997, Tridandapani et al 1997b). Furthermore, interactions between the TCR/CD3 complex phosphorylated immunoreceptor tyrosine based activation motifs (ITAMS) and SHIP have also been described (Osborne et al 1996). However whilst CD28 contains four potential sites for tyrosine phosphorylation (Y^{173}, Y^{188}, Y^{191}, and Y^{200}), none of these lie within any reognised ITAM or ITIM motif. Furthermore, in previous work published by this group it was not possible to demonstrate a physical association between SHIP and CD28. Given the lack of a recognised SHIP binding motif within the CD28 cytoplasmic tail, it is possible that CD28 couples to SHIP via intermediate adaptor molecules. Although an association between PI3K and a 5-phosphatase has been described in other systems (Liu et al 1996, Gupta et al 1999), it seems unlikely that PI3K is involved in the recruitment and activation of SHIP by CD28, since mutagenesis of the PI3K binding sites within the CD28 cytoplasmic tail had no effect on the ligation stimulated tyrosine phosphorylation of SHIP. Nevertheless the presence of proline rich and PTB domains within SHIP gives it potential for diverse biochemical interactions with a plethora of other proteins. In this respect there are at least two candidate adaptor proteins, namely Grb-2 and p62 Dok-1 which have been reported to co-associate with CD28 (Nunes et al 1996 and Schneider et al 1995) and which may therefore mediate an interaction between CD28 and SHIP.

Although the phosphorylation of SHIP, observed in response to CD28 and CD3 ligation indicates SHIP as a target for PTKs regulated by these signalling pathways, the significance of SHIP's function in CD28 and CD3 mediated signalling pathways has not yet been elucidated. Previous studies with $SHIP^{-/-}$ $Rag^{-/-}$ mice have reported normal TCR and CD28 -driven proliferation and IL-2 production (Liu et al 1998). The existence of alternative phosphatases may be able to substitute for SHIP in the SHIP-/- $Rag^{-/-}$ mice since SHIP is unlikely to be the only 5 -phosphatase with specificity for PI(3,4,5)P_3. For example the identification of the 5-phosphatase SHIP2 may explain the lack of severe defects in SHIP-/- mice, (Pesesse et al 1997). SHIP2 is the closest relative of SHIP, exhibiting around 38% overall identity, with

SHIP2 may explain the lack of severe defects in SHIP-/- mice, (Pesesse et al 1997). SHIP2 is the closest relative of SHIP, exhibiting around 38% overall identity, with 64% identity within the 5- phosphatase domain, and contains all the major structural features which have been described to be present in SHIP (Wisniewski et al 1999). SHIP2 has been described as a 155 kDa protein, and potentially antibody cross reactivity may mean that SHIP2 represents the 160 kDa band seen in whole cell SHIP immunoblots. SHIP and SHIP2 are not entirely redundant phosphatases however as, in contrast to SHIP, which can mediate the degradation of PI(3,4,5)P_3 and Ins(1,3,4,5)P_4, SHIP2s' substrate specificity is limited to PI(3,4,5)P_3 (Wisniewski et al 1999). In addition, whilst expression of SHIP is limited to haematopoietic and spermatogenic tissues, the wider expression pattern of SHIP2 protein may implicate it in the regulation of alternative phosphoinositide metabolism pathways to SHIP.

Furthermore the enzymatic action of the tumour suppressor PTEN has been described to mediate degradation of the PI3K product PI(3,4,5)P_3 through a 3-phosphatase activity (PTEN). Interestingly the phenotype of PTEN $^{-/-}$ mice is more severe than that of SHIP-/- mice, resulting in embryonic lethality (Di Cristofano et al 1998). In addition, the existence of 5- phosphatases which may regulate PI(3,4,5)P_3 accumulation and the accumulation of the lipid product of SHIP PI(3,4)P_2 in T cells have been described (Woscholski et al 1997). These 5-phosphatases share homology to PTEN through the amino acid sequence C(X)$_5$R at the active site (Zhang et al 1994).

Therefore the activation of SHIP by CD28 may act, in concert with other 5' poly phosphatases, and polyphosphatases outside of this family such as PTEN and 4-phosphatases as a negative feedback control on the CD28- activated PI3K dependent signalling cascade (Diagram 2). SHIP would facilitate the controlled removal of PI(3,4,5)P_3 and thus remove a membrane targeting signal for PH domain containing signalling molecules such as PKB and Tec kinases which are biochemical targets for CD28 (Parry et al 1997, Marengere et al 1997). A similar role for SHIP has been reported in the case of SHIP mediated inhibitory signalling cascades on PI(3,4,5)P_3 /Btk dependent signalling in B cells following FCγRIIB ligation (Bolland et al 1998 and Scharenberg et al 1998).

An alternative interpretation of the role of SHIP mediated degradation of PI(3,4,5)P_3 in CD28 driven signalling pathways can be given in which SHIP operates to shunt signalling away from PI(3,4,5)P_3 dependent effectors, towards effectors that are driven by PI(3,4)P_2, thus achieving diversity within the PI3K dependent signalling cascade (Diagram 2). This suggestion could explain the findings of one study which demonstrated that the catalytic activity of SHIP can play a positive role in IL-4 mediated signalling pathways (Giallourakis et al 2000). The notion that PI(3,4,5)P_3 and PI(3,4)P_2 exert different functional outcomes is contentious. For instance, while *in vitro* studies reveal that both these phosphoinositides exert similar potency for the binding of certain PH domains (Han et al 1998), other studies have revealed major differences in their effect on PKB activation (Alessi et al 1998, Franke et al 1997, Frech et al 1997, Klippel et al 1997). Thus it is unclear from *in vitro* studies whether distinct PI(3,4)P_2 driven pathways actually exist, although the situation *in vivo* may be quite different. However, in platelets, integrin ligation leads to an elevation in cellular levels of PI(3,4)P_2 and which correlates with the stimulation of PKB, without any increase in PI(3,4,5)P_3, and this supports the existence of pathways which are differentially regulated by these phosphoinositides (**Banfic** et al 1998). The notion that SHIP may facilitate the shunting of the PI3K dependent signalling pathways to downstream effectors differentially driven by D-3 phosphoinositides fits well with the array of functional responses attributable to PI3K. Such variance within a single signalling pathway may facilitate the numerous functional outcomes attributed to CD28, which include the regulation of T cell proliferation, IL-2 production, regulation of other cytokines/chemokines and their receptors as well as promotion of cell survival. Moreover, the growing repertoire of PH domain containing signalling molecules, that have been identified to mediate enzymatic or adaptor roles within the cell, implicate SHIP as a key regulator of CD28 driven functional events via the degradation of PI(3,4,5)P_3.

3.2.2 CTLA4 AND SHIP

CTLA4 has been previously demonstrated to associate with the p85 adaptor subunit of PI3K via the YVKM motif situated within the cytoplasmic tail of CTLA4 (Schneider et al 1995). Prior studies have demonstrated that ligation of CTLA4 with anti-CTLA4 antibodies resulted in an elevation of *in vitro* lipid kinase activity associated with anti- CTLA4 imunoprecipitates (Schneider et al 1995). Data presented here has demonstrated a marked *decrease* in cellular levels of PI(3,4,5)P_3,

in response to anti-CTLA4 ligation. Furthermore an elevation of PI(3,4)P_2 levels can be observed, which is roughly equal in magnitude to the decrease in PI(3,4,5)P_3. Initial comparison suggests that these data are in conflict with data presented by previous studies (Schneider et al 1995). However, fundamental differences in the techniques used by this study and those used by previous studies could explain the discrepancy in their findings.

Whilst the *in vitro* approach employed by Schneider *et al* allows the assignment of lipid kinase activity to specific protein complexes, the modulation of this activity observed in response to receptor ligation may be non-physiological. Differences between the data presented by this study and by Schneider may therefore be attributable to the following: Firstly, immunoprecipitation of proteins from whole cell lysates may exclude other proteins which in an intact cell would elicit receptor mediated regulation of lipid kinase activity. Secondly, the contamination of immunoprecipitates, by lipid kinases other than PI3K, may contribute to the lipid kinase activity measured. Thirdly, whilst the analysis of [^{32}P]- labelled phosphoinositide profiles from intact cells, as conducted by this study, can demonstrate more physiological patterns of receptor stimulated 3'-phosphoinositide accumulation, the extent of the contributory role played by particular lipid kinases or regulatory proteins can only be estimated and must therefore be further examined *in vitro*. Thus, whilst Schneider et al demonstrated that CTLA4 ligation, enhances PI3K activity measured *in vitro*, data described by this study suggests that the products of CTLA4 stimulated PI3K activity may be subject to negative regulation by lipid phosphatases. However, an alternative interpretation of these data would be that following ligation of CTLA4 PI3K activity leads to a dis-proportionate elevation of PI(3,4)P_2 as compared to levels of PI(3,4,5)P_3.

The analysis of anti-SHIP immunoprecipitates by this study, demonstrated that 145 kDa SHIP is a target for CTLA4 stimulated PTK activity. These data implicate SHIP in the regulation of D3-phosphoinositide levels following CTLA4 ligation. Previous studies have shown (Chacko et al 1996, Edmunds et al 1999) that the phosphorylation of SHIP correlates with elevated degradation of 5'-inositol poly-phosphatase activity. However, there is no evidence of a direct link between SHIP phosphorylation and upregulated phosphatase activity, as already discussed, and it may be that membrane localisation of SHIP may dictate activation. Furthermore the finding that SHIP may be activated in response to CTLA4 ligation may explain why this study failed to demonstrate any evidence for the CTLA4 mediated elevation

of PI(3,4,5)P_3 accumulation, despite the finding of previous studies which had shown that CTLA4 ligation could enhance PI3K activity *in vitro*. Taken together the data presented here indicate that upon CTLA4 ligation, SHIP becomes tyrosine phosphorylated which may mediate association with other proteins, facilitating membrane localisation and activation, and thus leading to the degradation of PI(3,4,5)P_3. Alternatively membrane localisation of SHIP, via a non phosphotyrosine dependent mechanism may precede tyrosine phosphorylation by membrane localised, CTLA4 activated, PTKs.

The coupling of SHIP to cellular proteins has been described within various signalling contexts including cytokine receptor signalling (Damen et al 1996) and TCR mediated signalling (Lamkin et al 1996) and may be considered to mediate its membrane approximation in the context of CTLA4 signalling. The cellular redistribution of SHIP may occur in a phosphotyrosine dependent manner through interaction with SH2 domain or PTB domain containing proteins. The association of SHIP with the PTB domain of Shc has been demonstrated in T cells (Lamkin et al 199) and is proposed to contribute to negative signalling in B cells through mediating the sequestration of Shc away from Grb2/Sos complexes (Tridandapani 1997c). SHIP may contribute to the CTLA4 inhibitory signal by mediating the recruitment of Shc away from Grb2.

Moreover, an interaction between CTLA 4 and the protein tyrosine phosphatase SHP2 has been described (Zhang et al 1997) which occurs via the association of the SH2 domain of SHP2 with the tyrosine phosphorylated cytoplasmic tail of CTLA4. It is thought that SHP2 can couple to SHIP via the SHIP SH2 domain (Sattler et al 1997). Hence upon recruitment of SHIP to the membrane by Shc or another PTB domain mediated interaction, SHIP may associate with SHP2 and directly couple to CTLA4. However this interaction would be dependent on the tyrosine phosphorylation of the intracytoplasmic domain of CTLA4. Interestingly recent studies have demonstrated that mutating the two sites of tyrosine phosphorylation (Y^{165} and Y^{182}) within the intracytoplamic tail of CTLA4 does not affect the inhibition of IL-2 secretion (Cinek et al 2000). Further work has demonstrated that although CTLA4 is known to associate with Src related kinases (Chuang et al 1999), the inhibition of ERK phosphorylation and IL-2 secretion are still able to occur in cell lines deficient in Zap-70 and lck (Baroja et al 2000) and thus inhibitory signalling is not dependent on the tyrosine phosphorylation of the CTLA4 tail by these PTKs. Thus the mechanism by which SHIP achieves membrane localisation

and activation following CTLA4 ligation is unlikely to be dependent on the tyrosine phosphorylation of CTLA4 by lck or Zap 70. Meanwhile tyrosine phosphorylation of CTLA4 at Y^{165} has been shown to be necessary for cell surface retention (Zhang et al 1997) and cell surface retention alone has been shown to be sufficient for CTLA4 to elicit an inhibitory effect, through the sequestration of B7.1 away from CD28, and thus the inhibition of stimulatory signalling pathways leading to IL-2 production (Baroja et al 2000). .....

CTLA4 functional models implicate this receptor in the control of T cell activation in resting cells and in previously activated CD8+ T cells. A model which has been proposed to describe CTLA4 mediated inhibition of naïve T cell activation suggest that, in the absence of T cell costimulation of sufficient magnitude, CTLA4's inhibitory effect predominates and inhibits downstream signalling pathways. Meanwhile in the presence of sufficient costimulatory signals, CD28 and/TCR/CD3 driven pathways are able to overcome CTLA4 inhibition and can lead to full scale T cell activation (Chambers et al 1999). The role of SHIP in such a model could be explained by the SHIP mediated degradation of PI(3,4,5)P_3 which has been described by the current study: insufficient co-stimulation could lead to the accumulation of a limited pool of PI(3,4,5)P_3 which would be rapidly removed through CTLA4 mediated enzymatic activation of SHIP. Meanwhile, in the presence of stronger costimulatory signals, SHIP may be unable to neutralise the larger pool of PI(3,4,5)P_3 generated by TCR/CD28 stimulation, thus allowing T cell activation to proceed.

SHIP has been implicated by this study in CD28 and CTLA4 signalling pathways, which have opposing downstream physiological effects. The earlier interpretation of the role of SHIP in the context of CD28 driven pathways considered that SHIP may provide a route for the negative feedback regulation of the CD28 signal. The suggestion that SHIP may also direct CD28 mediated signalling cascades towards PI(3,4)P_2 driven pathways, was also considered. In the absence of sufficient costimulatory signals, however, SHIP's predominant role in T cells may be downstream of CTLA4, where it is likely to act as a negative regulator of 3'-phosphoinositide accumulation, via PI3K, providing a natural braking mechanism for T lymphocyte costimulatory pathways.

3.2.3 3'-PHOSPHOINOSITIDE REGULATION IN THE LEUKAEMIC CELL LINE-JURKAT

Measurement of 3' -phosphoinositides in Jurkat T cells by this study has indicated high basal levels of PI(3,4,5)P_3 and PI(3,4)P_2, in comparison to the levels of these lipids that are detectable in the A20 B cell lymphoma cell line. The causative factors that may contribute to the basal 3'-phosphoinositide levels in different cell lines are considered here:

Firstly, the absence of metabolic breakdown of 3'-phosphoinositides by lipid phosphatases may be responsible for the basal levels of these lipids in a particular cell line. Secondly, the basal levels of PI(3,4,5)P_3 measured in a cell line may be a direct result of the constitutive activity of PI3K in those cells. Thirdly, the combined levels of lipid phosphatase expression **and** basal PI3K activity, may each contribute to the resting 3'phosphoinositide levels in a given cell line.

In an attempt to determine which of these scenarios may apply in Jurkats and A20 cells, this study has demonstrated that the acute lymphoblastic leukaemic T cell line Jurkat lacks two major routes for degradation of PI(3,4,5)P_3, via the 3'-phosphatase PTEN (Shan et al 2000) and the inositol (poly)phosphate 3'-phosphatase SHIP. Thus the lack of degradative pathways for 3'phosphoinositides may be responsible for their high basal levels in Jurkats. In support of this hypothesis, this study has described data which demonstrates the constitutive membrane recruitment of GFP-PH-PKB which results due to the high basal levels of PI(3,4,5)P_3 in Jurkats. Interestingly the overexpression of catalytically active SHIP can mediate the redistribution of GFP-PKB-PH to the cytosol. These constructs expressed amino acids 364-825 of 145kDa SHIP. The minimal catalytic core of SHIP has recently been described as amino acids 400-866, and constructs which lacked amino acids 450-466 were insufficient to mediate 5' phosphatase activity in vitro (Aman et al 2000). Data described in results section 3.2 of this study describes the *in vitro* lipid phosphatase activity mediated by rCD2 SHIP and thus proposes that the minimal catalytic core of SHIP is smaller than that proposed by Aman and co-workers. Indeed, the redistribution of GFP-PKB-PH from the membrane, elicited by rCD2-SHIP also indicates, although does not unequivocally prove, that this region of the SHIP protein is sufficient to mediate 5' -phosphoinositide activity *in vivo*. This data further suggests that the absence of pathways for the enzymatic degradation of PI(3,4,5)P_3 in Jurkats may be responsible for the high basal levels of this lipid.

In contrast to J6 the A20 B cell lymphoma cell line expresses readily detectable levels of SHIP and lower levels of PTEN. Thus, the existence of degradative

pathways for PI(3,4,5)P_3 metabolism may reduce basal levels of this lipid in A20s. Alternatively low levels of 3'-phosphoinositides in A20 cells may correlate with nominal basal levels of PI3K activity. Interestingly, the basal level of PI(3,4)P_2 measured in A20 cells is twice that of PI(3,4,5)P_3, which may indicate that PI3K is basally active, but that its products are subject to negative regulation through 5'-phosphatase activity, leading to a disproportionate accumulation of this lipid. Basally active PTEN 3'-phosphatase activity would also lower PI(3,4,5)P_3 levels, however it is difficult to detect the accumulation of PI(4,5)P_2, the product of PI(3,4,5)P_3 metabolism by PTEN, as cellular levels of this phosphoinositide are constitutively high in all cell types. Thus any accumulation of PI(4,5)P_2 may make an undetectable change to the overall cellular pool. Alternatively basally active PI3K, in A20 cells may preferentially phosphorylate PI(3)P over PI(4,5)P_2, leading to the accumulation of a larger cellular pool of PI(3,4)P_2 than of PI(3,4,5)P_3.

Work carried out more recently by this laboratory has also demonstrated that the basal activation of the PI3K effector PKB, and phosphorylation of the PKB target GSK-3, in resting Jurkats (personal communication Steven Burgess), correlate closely with 3'-phosphoinositide levels detected by this study. Previous studies have demonstrated that the *in vitro* kinase capacity of epitope tagged PKB could be upregulated following antigen receptor or CD28 stimulation (Parry et al 1997) of Jurkat cells. However more recent work has indicated that endogenous PKB is constitutively activated and that endogenous levels of PI(3,4,5)P_3 are sufficient to saturate the PI3K/PKB pathway and thus uncouple it from antigen receptor regulation (Steven Burgess, personal communication). In accordance with these findings work by other groups has examined the basal states of other targets for PI3 K produced 3'-phosphoinositides in Jurkat T cells. It has been shown that Tec kinase ITK is constitutively active in Jurkats (Shan et al 2000) and that the actin binding cytoskeletal protein cofilin, which is regulated by costimulatory signals in peripheral blood lymphocytes, is constitutively dephosphorylated, and is basally associated with actin in resting Jurkat cells (Lee et al 2000).

Taken together these data suggest that constitutively high levels of PI3K activity are displayed by Jurkat T cells, and this coupled to the lack of lipid phosphatase expression by this cell line would contribute to 3'-phosphoinositide levels in these cells.

Data presented in this study demonstrate that the inhibition of PI3K in Jurkat T cells, by treatment of cells with LY249002, can elicit the re-distribution of GFP- PKB - PH from the membrane into the cytosol. However the kinetics of this redistribution are extremely slow, with full cytoplasmic re-distribution occurring after 1 hour. Furthermore LY249002 treatment of Jurkats results in the de-phosphorylation of endogenous PKB and its downstream target, GSK-3, which occurs with similarly delayed kinetics (personal communication Steven Burgess). Thus it may be concluded from this data that the high basal level of 3'phosphoinositides in the Jurkat cell line may not be solely dictated by basally elevated PI-3K activity, but may also be related to rates of lipid degradation.

Previous studies in the A20 cell line (Astoul et al 1999) have shown that LY249002 treatment of cells expressing constitutively active PI3K causes the redistribution of GFP-PKB-PH from the membrane to the cytosol within 30 seconds. Further analysis of events downstream of PI3K, demonstrated that PKB activation and GSK-3 phosphorylation are also rapidly attenuated, following LY249002 treatment administered 5 minutes after $F(ab)_2$ treatment (personal communication, Emmanuelle Astoul). It may be considered that the basal activities of SHIP and PTEN in the A20 cell line may facilitate the rapid turnover of PI(3,4,5)P_3 produced by basally active endogenous PI3K activity, and thus following the inhibition of constitutively expressed PI3K, 3'- phosphoinositides produced prior to PI3K inhibition are rapidly depleted by the basal activities of SHIP and PTEN.

It appears that in the cell lines considered by this study both constitutively active PI3K and the presence or absence of lipid phosphatases, act in concert to positively and negatively regulate basal levels of 3'phosphoinositides repectively. This conclusion may have important connotations for the interpretation of previous data which has described the failure of PI3K inhibitors to block T cell costimulatory responses in Jurkat T cells (Lu et al 1995). The findings of this study indicate that as Jurkat cells lack two important inositol(poly)phosphate phosphatases and require prolonged treatment with PI3K inhibitors to suppress PI3K responses and create lower cellular pools of 3'-phosphoinositides. This means that prior studies which have employed the use of PI3K inhibitors in the Jurkat T cell line may have underestimated the role of PI3K in CD28 mediated signalling. Furthermore, it would be interesting to assess primary T cell leukaemias which may possess similarly elevated levels of PI3K effectors and elevated pools of 3'-

phosphoinositides. In this respect, loss of function mutations of the PTEN gene locus have been identified in many human cancers (Cantley et al 2000), and a link between this 3'-phosphatase and the PI3K/PKB pathway is now widely accepted (Wu et al 1998, Myers et al 1998).

3.2.4 - CONCLUSIONS

This study has demonstrated that the tyrosine phosphorylation of SHIP is elicited in response to physiological stimulation of the CD28 receptor by its ligand B7.1. This finding implicates SHIP in the regulation of PI(3,4,5)P_3 accumulation in T cells in response to costimulatory signals. Furthermore, simultaneous ligation of CD3 and CD28 induces additive tyrosine phosphorylation of SHIP which suggests that SHIP may mediate PI(3,4,5)P_3 accumulation downstream of both of these receptors. It is proposed that SHIP may control a negative feedback pathway which acts to dampen signals leading to T cell activation, or that it shunts signalling towards PI(3,4)P_2 driven pathways (see diagram 14). The physical relationship between SHIP and CD28 is unclear, but does not appear to be dependent on PI3K association with CD28, or the activity of PI3K.

CTLA4 ligation elicits a reduction in the basal levels of 3'-phosphoinositides present in unstimulated T cells. This study has shown that the tyrosine phosphorylation of SHIP is also induced in response to CTLA4 stimulation, and thus SHIP's catalytic activity may contribute to the inhibitory signal mediated by this receptor.

The leukaemic T cell Jurkat exhibits high basal levels of PI(3,4,5)P_3 and PI(3,4)P_2, which is considered to be due to high basal levels of PI3K activity in this cell line and the lack of inositol phosphatase expression. Furthermore this study has shown that the turnover of PI(3,4,5)P_3, following inhibition of PI3K with LY249002, occurs with delayed kinetics, and it is therefore proposed that previous studies which have used PI3K inhibitors in this cell line may have misrepresented the role of PI3K in CD28 mediated signalling pathways.

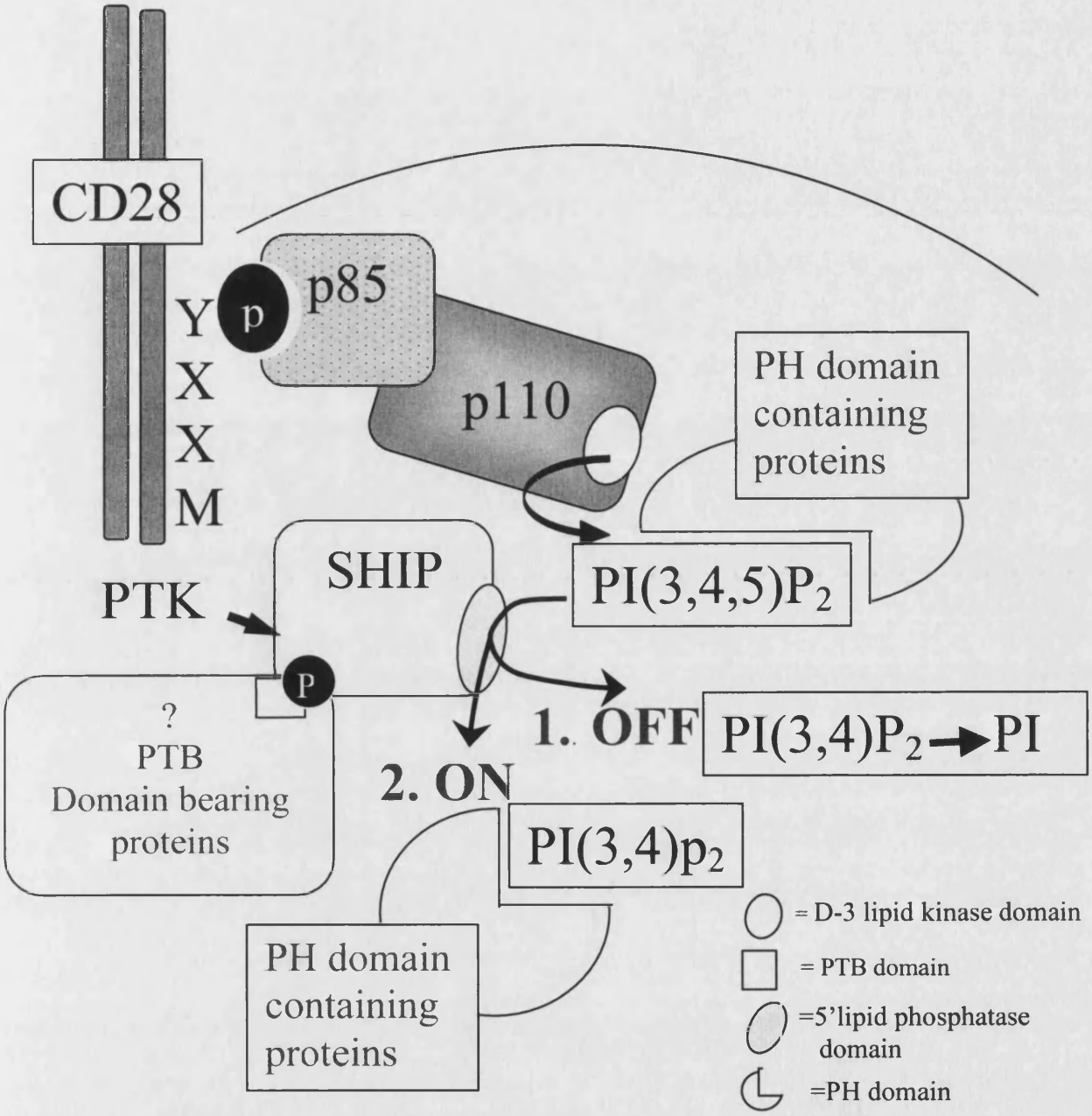


Diagram 16: Schematic depiction of CD28 mediated tyrosine phosphorlyation of SHIP.

Tyrosine phosphorylationof SHIP is mediated by CD28 in response to ligation by B7.1.Tyrosine phosphorylation of SHIP is thought to mediate association of SHIP with Shc via the SHIP NPXPY binding to the PTB domain of Shc. The SHIP mediated enzymatic degradation of the PI-3 K metabolic $PI(3,4,5)P_3$ may act as a negative feedback loop to attenuate PI-3 K mediated CD28 regulated signalling (1). Alternatively, the product of SHIPs' enzymatic activity $PI(3,4,)P_2$ may act to shunt signalling towards as yet unidentified proteins whos PH domains bind $PI(3,4)P_2$ (2).

3.3 REGULATION OF PI3K DEPENDENT SIGNALLING CASCADES IN B LYMPHOCYTES

3.3.1 FCγRIIB MODULATION OF BCR MEDIATED SIGNALLING CASCADES IN A20 CELLS.

The following section of this study describes work carried out in the Balb/c B lymphoma cell line A20. This work was undertaken due to the existence of well characterised pathways, regulated by the BCR and dependent on PI3K, in this cell line. These pathways lead to calcium mobilisation, cell survival, antibody production, and proliferation (see Introduction), and are subject to negative regulation following co-ligation of the FCγRIIB with the BCR. The inositol-(poly) 5'-phosphatase SHIP, which already described by this study to play a role in T lymphocyte signalling (see results), has been demonstrated by Scharenberg and others to be a pivotal effector of the FCγRIIB mediated signal (Scharenberg et al 1998, Nakamura et al 2000). Thus the following section describes research which has examined the regulation of PI3K dependent signalling pathways in the B cell line A20.

Crosslinking the BCR in the A20 cell line is achieved using a F(ab')2 fragment of rabbit anti-mouse IgG, hereafter referred to as (Fab')2 of RAMIG. Meanwhile, BCR co-ligation with the FCγRIIB is accomplished using intact rabbit anti-mouse IgG (RAMIG). Used in equimolar amounts, comparison of the effects of F(ab')2 and intact RAMIG treatment of A20 cells facilitates the dissection of stimulatory and inhibitory signalling cascades in this B cell model.

FCγRIIB MODULATION OF BCR INDUCED CALCIUM FLUX.

Many studies have shown that FCγRIIB co-ligation with the BCR inhib BCR mediated calcium flux, in a PI3K dependent manner (Ono et al 1998, Bolland et al 1998). To examine the effects of BCR ligation or BCR/FCγRIIB co-ligation on calcium mobilisation in A20 cells, cells were loaded with Fura-2AM and stimulated as described in figure 25. Calcium responses were measured by fluorimetry (Figure 25) using the fluorescent probe fura-2AM. This experiment served to demonstrate that activating and inhibitory stimulation of signalling pathways, leading to calcium mobilisation, could be achieved in this cell line.

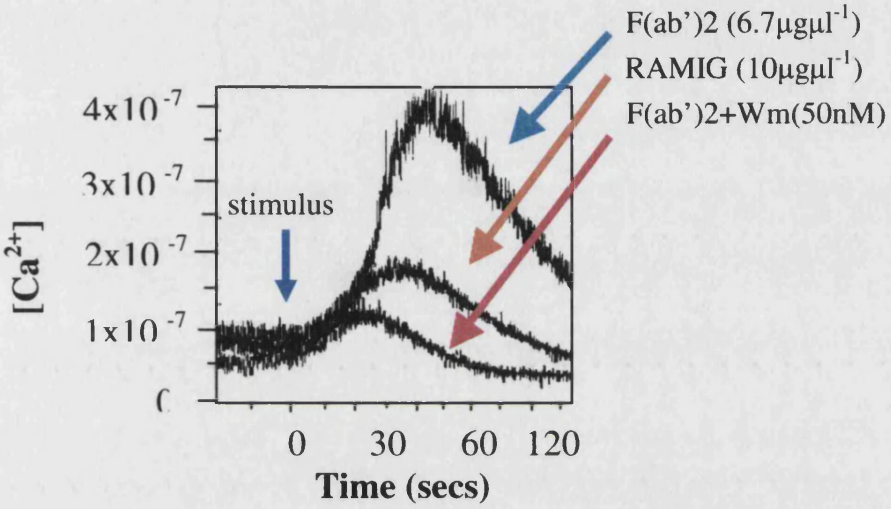


Figure 25: FCγRIIB1 and wortmannin mediated impairment of BCR elicited calcium responses in FURA 2-AM loaded A20 cells.

A20 cells were loaded with Fura-2AM for 30 mins at 37°C. After washing in Ca^{2+} Mg^{2+} free HBSS, cells were resuspended in same at $1x10^6$ cells ml^{-1}. 1ml aliquots of the labelled cell suspension were either stimulated at 37°C with, 24 µg F(ab')2 fragments of RAMIG or 40 µg RAMIG; alternatively cells were pre-treated at 37°C with wortmannin and then stimulated with F(ab')2 of RAMIG. Calcium mobilisation was measured via fluorimetric analysis. These data are from a single experiment and are representative of six separate experiments.

BCR ligation mediated a rapid initial phase calcium elevation followed by a gradual return to basal. FCγRIIB co-ligation with the BCR suppressed the initial phase calcium spike, and almost completely abrogated the late phase response (figure 25). Interestingly, the PI3K inhibitor wortmannin was able to inhibit BCR mediated calcium mobilisation, in a manner similar to FCγRIIB mediated inhibition of this response (figure 25).

FCγRIIB MODULATION OF BCR INDUCED ERK1/2 PHOSPHORYLATION

FCγRIIB inhibition of BCR mediated Ras activation (Sarmay et al 1996), and Raf1 (Moodie et al 1996) and ERK phosphorylation (Campbell et al 1995) has been reported. To confirm that BCR and FCγRIIB co-ligation in the A20 cell line either optimally stimulated or inhibited respectively, pathways leading to ERK phosphorylation, cells were treated with F(ab')2 and RAMIG. Whole cell lysates were prepared for immunoblot analysis using anti-phospho ERK1/2 antibodies.

BCR ligation with F(ab')2 fragments, stimulated the rapid phosphorylation p44/42 kDa proteins (figure 26) which was diminished following BCR/FCγRIIB co-ligation when cells were treated with RAMIG (figure 26). Re-probing with anti-pan ERK1/2 confirmed that the p44/42 kDa proteins were ERK1/2 and demonstrated equal loading and transfer of proteins (figure 26).

FCγRIIB MEDIATED MODULATION OF PI3K ACTIVITY. ASSOCIATION OF p85 WITH CD19

Previous studies have demonstrated that BCR ligation results in the CD19 mediated recruitment of PI3K (Tuveson et al 1993), and that this can be inhibited by FcγRIIB co-ligation with the BCR (Kiener et al 1996). In order to verify that F(ab')2 and RAMIG stimulation of the A20 cell line couples the BCR and FCγRIIB efficiently to the regulation of PI3K recruitment, anti-p85α immunoblot analysis of anti-CD19 immunoprecipitates was performed. This demonstrated the rapid recruitment of p85 to CD19 which was sustained for 10 minutes following ligation (figure 27). This co-association diminished to sub basal levels following co-ligation of the FCγRIIB with the BCR. Re-probing with anti-CD19 demonstrated equal loading of anti CD19 immunoprecipitated protein (figure 27).

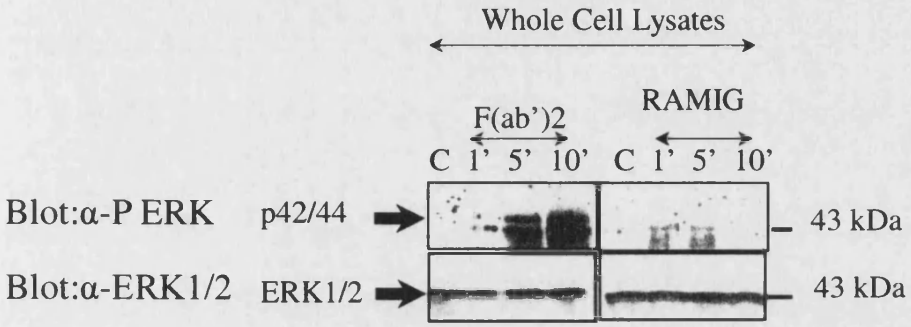


Figure 26: FCγRIIB modulation of BCR induced ERK1/2 phosphorylation in A20 cells.

$2x10^6$ A20 cells per point were either left unstimulated as a control or stimulated via the BCR, with 12 μg F(ab')2 fragments of RAMIG, or via FCγRIIB co-ligation with the BCR via 20μg RAMIG. Cells were lysed and subject to acetone presipitation on ice for 1 hour. After centrifugation proteins were boiled in SDS-Laemmli sample buffer and separated via SDS-PAGE. Proteins were then transferred onto nitro-cellulose for immunoblotting with anti-phospho-ERK1/2 at 1 μg per ml in 0.05% marvel/PBS. Blots were subsequently stripped and re-probed with anti-PAN ERK1/2 to verify equal loading of protein and transfer. These data are from a single experiment and are representative of four separate experiments.

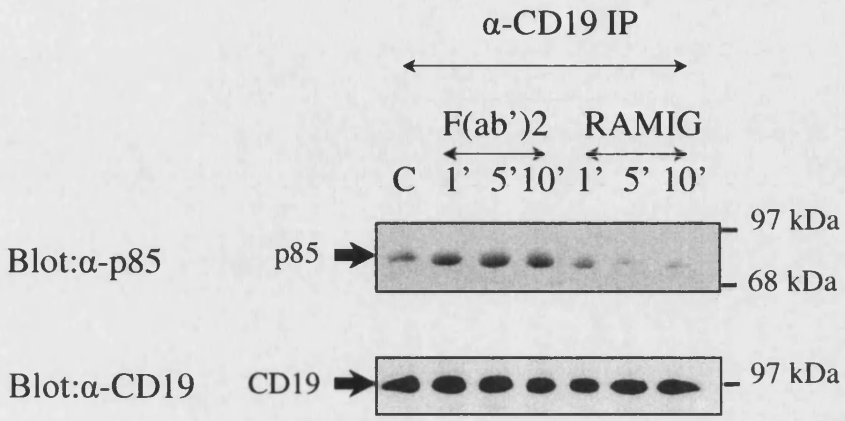


Figure 27: BCR ligation versus BCR/FCγRIIB1 co-ligation induced co-association of p85 with CD19.

$2x10^7$ A20 cells were either left unstimulated as a control or stimulated with 20 μg RAMIG or 12 μg F(ab')2 fragments of RAMIG. Cells were lysed and immunoprecipitated with 1 μg anti-CD19. Precipitates were separated by 7.5 % SDS-PAGE and transferred onto nitro-cellulose for immunoblotting with anti-p85 (top panel) at a concentration of 1 μg per ml in 0.05 % marvel/PBS. Blots were then stripped and re-probed with anti- CD19 (1 μg per ml 0.05 % marvel/PBS) to verify equal loading of immunoprecipitated protein (bottom panel). These data are from a single experiment which are representative of four experiments.

ACCUMULATION OF 3'-PHOSPHOINOSITIDE LIPIDS IN RESPONSE TO BCR LIGATION AND BCR/FCγRIIB CO-LIGATION.

Data described so far, in this section, has demonstrated that efficient activation or inhibition of pathways leading to ERK activation, calcium mobilisation and the recruitment of PI3K to CD19 in A20 cells can be elicited by ligation of the BCR and coligation of the FCγRIIB. In addition it was important to demonstrate that the BCR mediated association of PI3K with CD19 was accompanied by the accumulation of D-3 lipids which could be inhibited by coligation of the FCγRIIB, the accumulation of PI(3,4,5)P_3 following F (ab')2 of RAMIG or RAMIG stimulation was measured. Cells were labelled with $^{[32]}$P- orthophosphoric acid and changes in cellular 3'-phosphoinositide levels were measured following extraction and deacylation of lipids via HPLC separation.

As can be seen in figure 28, F(ab')2 mediated stimulation of the BCR resulted in a gradual accumulation of PI(3,4,5)P_3 at one minute post stimulation which had risen by over 7- fold from 421 cpm to 3004 cpm at 15 minutes. In contrast BCR/FcγRIIB1 co-ligation, produced a slight increase in PI(3,4,5)P_3 levels which peaked at 694cpm, a 1.6 fold rise at 10 minutes. Stimulation of the BCR resulted in the initial accumulation of PI(3,4)P_2 which peaked at 5 minutes at 1279 cpm and which represented a 3 fold increase above basal levels (figure 28). RAMIG stimulation of the BCR/FCγRIIB resulted in the initial accumulation of PI(3,4)P_2 which peaked after 5 minutes at 814 cpm (figure 28).

The activation of SHIP following FCγRIIB co-ligation has been previously described to regulate levels of PI(3,4,5)P_3 (Scharenberg et al 1998), leading to the production of the enzymatic product of SHIP PI(3,4)P_2. However, although RAMIG stimulation of the BCR/FCγRIIB, lead to the marked inhibition of PI(3,4,5)P_3 accumulation, there was no concomittant increase in PI(3,4)P_2 at later time points (figure 28). It therefore appeared that BCR ligation in the A20 cell line rapidly stimulated PI3K activation, leading to the accumulation of PI(3,4,5)P_3 and PI(3,4)P_2, and that whilst the accumulation of PI(3,4,5)P_3 could be abolished by BCR/FCγRIIB co-ligation the evidence of SHIP mediated degradation of this lipid to PI(3,4)P_2 not be seen.

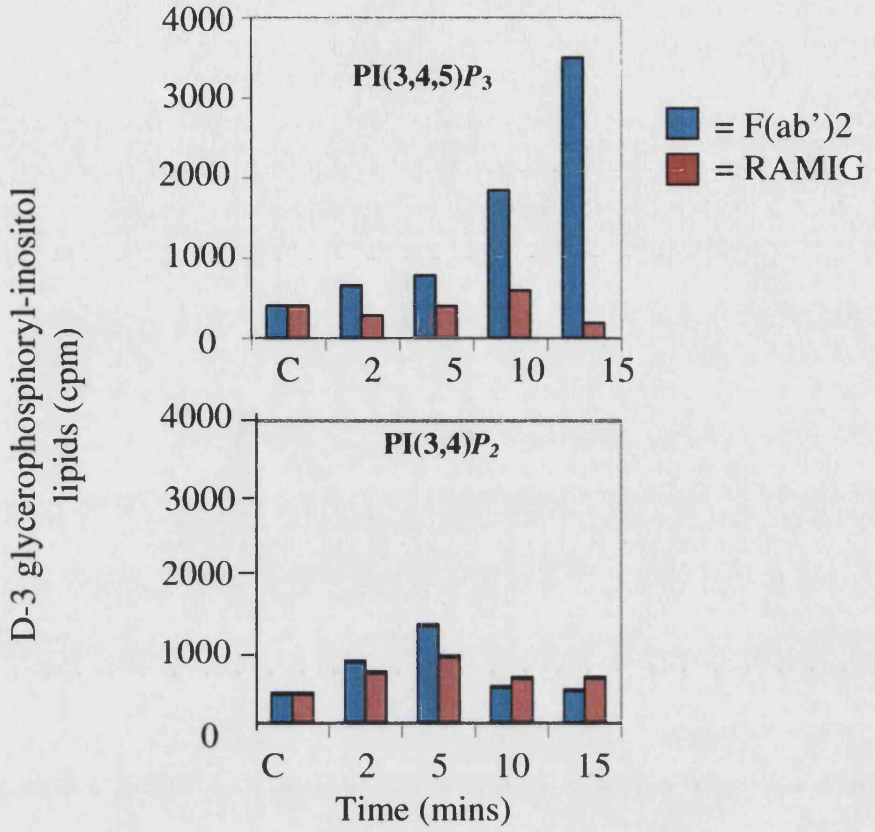


Figure 28: FCγRIIB modulation of BCR mediated PIP3 production.

A20 cells were labelled with [^{32}P]- phosphoric acid for five hours at 37 ^{0}C and then either pretreated with 100nm wortmannin or left untreated. 20x10^6 cells per point were either left un-stimulated as a control or stimulated with 20 μg RAMIG or 12 μg F(ab')2 fragments of RAMIG. Cells were then lysed and lipids were extracted. lipids were deacylated and analysed by HPLC using a partisphere Sax column.
Total counts: PI(3,4,5)P_3=1,537,000cpm. PI(3,4)P_2= 9997,567 cpm.These data are from a single experiment and are representative of three separate experiments.

TYROSINE PHOSPHORYLATION OF SHIP IN RESPONSE TO BCR LIGATION AND BCR FCγRIIB CO-LIGATION.

SHIP has been described as a major component of the FCγRIIB inhibitory signal (Scharenberg et al 1998, Nakamura et al 2000). To examine the effects of BCR ligation and BCR/ FCγRIIB 1 co-ligation on SHIP, tyrosine phosphorylation in response to F(ab')2 and RAMIG treatment was examined. SHIP proteins were immunoprecipitated from resting and stimulated cell lysates and blotted with 4G10 (Figure 29). Multiple tyrosine phosphorylated bands could be seen in SHIP immuno-precipitates (figure 29). The most heavily phosphorylated of which was 145 kDa band which was most prominent at 1 minute post stimulation, and upon re-probing (figure 29) was confirmed to be 145 kDa SHIP. However at 1 minute the tyrosine phosphorylation of a lower band could be seen. The identity of the additional bands was not known, however it is possible that these are SHIP related proteins or different isoforms of SHIP which could be co-precipitated by the anti-SHIP polyclonal antiserum (see discussion section 3.2).

SUMMARY

1) FCγRIIB co-ligation with the BCR, through the treatment of A20 cells with intact RAMIG was demonstrated to inhibit F(ab')2 stimulation of BCR mediated calcium mobilisation.
2) Wortmannin treatment of cells prior to BCR ligation with F(ab')2 fragments, abrogated calcium mobilisation and demonstrated the major role of PI3K in pathways leading to calcium mobilisation.
3) BCR ligation stimulates the gradual production of PI(3,4,5)P_3 peaks at 15 minutes, and is inhibited following FCγRIIB co-ligation with the BCR.
4) BCR ligation leads to the accumulation of PI(3,4)P_2 which peaks at 5 minutes and diminishes thereafter. PI(3,4)P_2 accumulation is partially inhibited following FCγRIIB co-ligation.
5) Tyrosine phosphorylation of SHIP occurs rapidly upon co-ligation of the BCR and FCγRIIB receptors, and is sustained for up to ten minutes. Ligation of the BCR alone mediates the moderate, yet reduced tyrosine phosphorylation of SHIP.

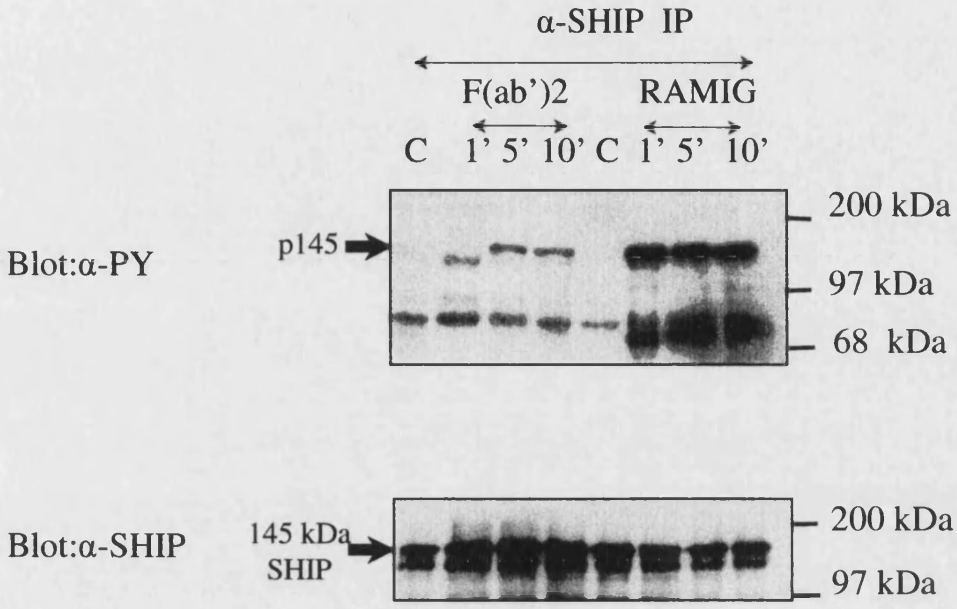


Figure 29: BCR ligation versus BCR/FCγRIIB1 co-ligation stimulated tyrosine phosphorylation of anti-SHIP immunoprecipitates.

$2x10^7$ A20 cells were either left unstimulated as a control or stimulated with either 20 μg RAMIG or 12 μg F(ab')2 fragments of RAMIG. Cells were lysed and precipitated with 1 μg anti-SHIP. Precipitates were separated by 7.5 % SDS-PAGE and transferred onto nitro-cellulose for immunoblotting with anti-phosphotyrosine antibody 4G10 (top panel) at a concentration of 1 μg per ml in 0.05% marvel/PBS. Blots were stripped and re-probed with anti SHIP to verify equal loading of immuno-precipitated protein. These data are from a single experiment and are representative of more than six others.

3.3.2 FCγRIIB MODULATION OF PI3K RECRUITMENT TO TYROSINE PHOSPHORYLATED PROTEIN COMPLEXES

It was postulated that in addition to upregulating the enzymatic activity of SHIP, FCγRIIB co-ligation may negatively regulate the accumulation of PI3K lipid products in A20 cells by controlling the sub-cellular localisation of PI3K proteins. Inhibitory signalling cascades, initiated by FCγRIIB co-ligation with the BCR, may mediate the molecular interaction of PI3K, via the SH2 domains of the p85 adaptor subunit, with a distinct array of tyrosine phosphorylated complexes within the cell. Recruitment of PI3K to such complexes might be less conducive to PI3K activation, or may localise PI3K activity away from $PI(3,4,5)P_3$ dependent signalling pathways and into the proximity of SHIP. The working hypothesis for these experiments was that PI3K is differentially recruited to tyrosine phosphorylated protein complexes in response to BCR and FCγRIIB stimulation. To address this possibility comparative levels of lipid kinase activity, associated with anti-phospho tyrosine immunoprecipitated proteins following BCR and BCR FCγRIIB co-ligation, was examined.

A20 cells were either left unstimulated or stimulated with F(ab')2 and RAMIG, and subject to immunoprecipitation with anti-phoshotyrosine antibody, 4G10. Immunoprecipitates were then assayed *in vitro* for lipid kinase activity. Enhanced levels of lipid kinase activity were immunoprecipitated with tyrosine phosphorylated complexes following co-ligation of the FCγRIIB with the BCR than following ligation of the BCR alone (figure 30).

TYROSINE PHOSPHORYLATION OF WHOLE CELL EXTRACTS FOLLOWING BCR LIGATION AND BCR/FCγRIIB COLIGATION

To further define the nature of the FCγRIIB mediated inhibitory signal in B cells this study has examined the protein targets of BCR and FCγRIIB mediated protein tyrosine kinase activity. Previously it has been observed that co-crosslinking the FCγRIIB with the BCR does not markedly reduce tyrosine phosphorylation of cellular proteins (Sarkar et al 1995). Several proteins, namely the FCγRIIB and SHIP (Kiener et al 1997) have been shown to exhibit higher levels of tyrosine phosphorylation upon FCγRIIB coligation with the BCR. Meanwhile the phosphorylation of other proteins is decreased e.g.: PLCγ2 (Sarkar et al 1996), and CD19 (Tuveson et al 1996). Interestingly, the PTK's Lyn and Syk are activated

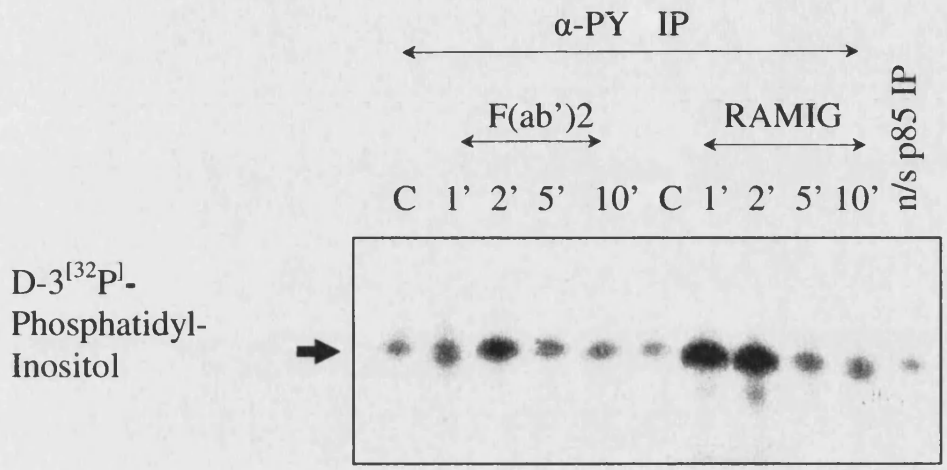


Figure 30: F(ab')2 and RAMIG induced *in vitro* lipid kinase activity associated with anti-phospho tyrosine immuno-precipitates.

2 x10^7 A20 cells were either left unstimulated as a control or stimulated with either 20 μg RAMIG or 12 μg F(ab')2 fragments of RAMIG. Cells were lysed and precipitated with 1 μg anti-phosphotyrosine antibody 4G10. As a positive control 2 x10^7 cells were lysed and immunoprecipitated 1 μg with anti-p85. Precipitates were incubated *in vitro* with phosphatidyl-inositol and [^{32}P]- ATP to assay lipid kinase activity. Nascent phospho-lipids were separated by thin layer chromatography and detected via autoradiography. These data are from a single experiment and are representative of three experiments.

following both BCR ligation and BCR/FCγRIIB co-ligation and reports have described Lyn in direct association with the FCγRIIB ITIM (Sarkar et al 1996). In the first instance this study sought to illustrate the tyrosine phosphorylation triggered following either BCR ligation or BCR FCγRIIB co- ligation via anti-phosphotyrosine immunoblot analysis of whole cell lysates stimulated with F(ab')2 fragments of RAMIG or RAMIG respectively.

Tyrosine phosphorylation of proteins which migrated at 145 kDa, 100 kDa, 70 kDa and 62 kDa protein were identified following BCR ligation (figure 31). Phosphorylation of p145, and p100 was elevated in response to BCR FCγRIIB coligation, as compared to BCR ligation alone. In contrast tyrosine phosphorylation of p70 was higher in response to BCR ligation alone, and was induced at only low levels in response to BCR/FCγRIIB co-ligation (figure 31). The tyrosine phosphorylation of all proteins, in response to BCR ligation and BCR/FCγRIIB co-ligation, was rapid with maximal phosphorylation being detected after one minute (figure 31). The putative identification of these proteins is addressed in subsequent sections.

DIFFERENTIAL TYROSINE PHOSPHORYLATION OF GAB 2 FOLLOWING LIGATION OF THE BCR OR COLIGATION OF THE BCR/FCγRIIB.

One possible identity of the 100 kDa protein in A20 whole cell lysates, which was subject to tyrosine phosphorylation in response to BCR ligation or co-ligation of the BCR/FCγRIIB, was putatively identified as the adaptor protein Gab2. Studies in haemopoietic cells (Craddock and Welham 1997) and T cells (Nishida et al 1999), have examined the receptor triggered phosphorylation of a 100 kDa band which reportedly co-precipitates with SHP2, PI3K, Grb-2. Multiple tyrosine phosphorylation sites exist within the amino acid sequence of this protein which in phosphorylated state can be seen as an electrophoretic band shift. More recently this protein has been cloned and named Gab2 (Nishida et al 1999).

To investigate whether the 100 kDa phospho-protein seen in stimulated A20 cell lysates was in fact Gab 2, immunoprecipitates were prepared using Gab 2 antibodies from resting and RAMIG or F(ab')2 of RAMIG stimulated A20 cell lysates (Figure 32). Immunoblotting with anti-phosphotyrosine antibody 4G10 is shown in the top panel and revealed the tyrosine phosphorylation of a 100 kDa protein. Re-probing

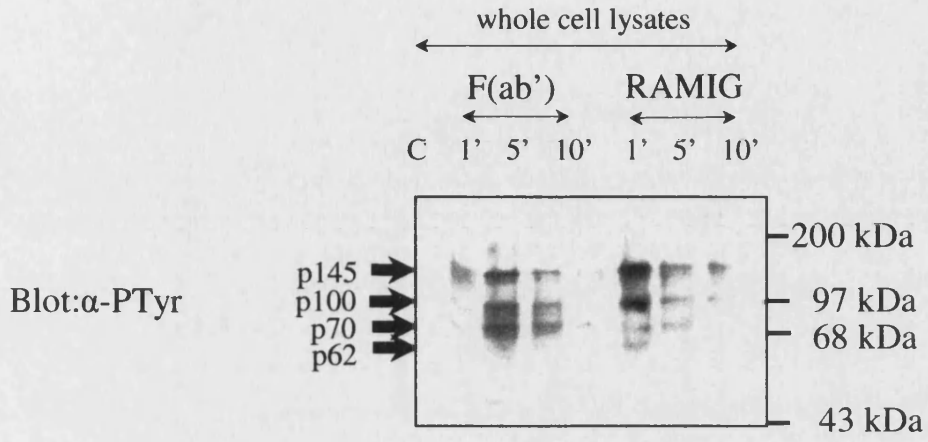


Figure 31: Modulation of BCR induced tyrosine phosphorylation of cellular proteins, following BCR/FCγRIIB co-ligation.

$2x10^6$ A20 cells were either left unstimulated as a control or stimulated with 20 μg RAMIG or 12 μg F(ab')2 fragments of RAMIG. Cells were lysed and proteins were acetone precipitated and boiled in SDS-laemmli sample buffer. Proteins were then separated by SDS-PAGE and transferred onto nitrocellulose for immunoblotting with anti phosphotyrosine antibody 4G10 at a concentration of 1 μg per ml in 0.05% marvel/PBS. Approximate sizes of phospho-tyrosyl proteins are indicated on the left of the figure. These data are from a single experiment representative of three others representative of three others.

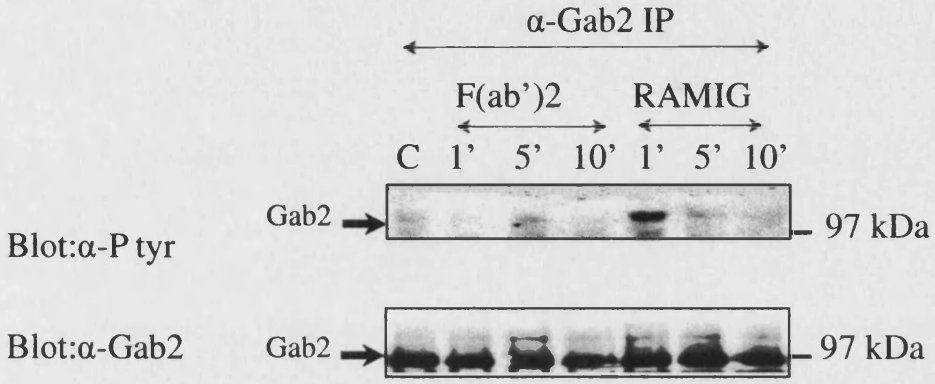


Figure 32: RAMIG and F(ab')2 induced tyrosine phosphorylation of Gab2 immunoprecipitates.

$2x10^7$ A20 cells were either left unstimulated, as a control or stimulated with 20 μg RAMIG or 12 μg F(ab')2 fragments of RAMIG. Cell lysates were immuno-precipitated with 1 μg anti-Gab2 and proteins were separated by 7-17 % SDS-PAGE and transferred to nitro-cellulose for immunoblotting with anti-phosphotyrosine antibody 4G10 (upper panel) at a concentration of 1 μg per ml in 0.05 % marvel/PBS.

Blots were stripped and re-probed with anti- Gab2, at 1 μg per ml, to verify equal loading of immunoprecipitated protein (lower panel). These data are from a single experiment representative of three others.

with anti-Gab2 (*lower panel*, Figure 32) confirmed that this protein was Gab 2 and illustrated that a band shift occurs upon stimulation induced tyrosine phosphorylation of Gab 2. Moreover it appeared that Gab 2 was more strongly tyrosine phosphorylated following co-ligation of BCR/FCγRIIB than by ligation of the BCR alone (Figure 32). The identification of the differential phosphorylation of Gab 2 by BCR and BCR/FcγRIIB mediated signalling pathways, further characterises the early biochemical events which mediate the inhibitory signalling cascade.

BCR LIGATION AND BCR/FCγRIIB CO-LIGATION STIMULATES THE PRESENCE OF GAB2 IN ANTI p85 PRECIPITATES.

This study has demonstrated that the p85 adaptor sub-unit of PI3K can associate strongly with CD19 following BCR ligation, and that this interaction leads to the activation of PI3K and the production of PI(3,4,5)P_3 (Tuveson et al 1993, Gold et al 1994). This event plays a pivotal role in the triggering of the upstream events in B cell activation. In addition to CD19, the docking protein Gab2 also contains p85 SH2 binding motifs (Nishida et al 1999). The presence of these motifs within the Gab2 protein led to the hypothesis that Gab2 and p85 may associate in A20 cells, as they have been described to do in numerous previous studies in haemopoietic cells (Craddock and Welham 1997, Carlberg et al 1997, Nishida et al 1999). Initially it was important to identify whether a co-association between p85 and Gab2 following BCR ligation or FCγRIIB co-ligation in A20 cells. p85 immunoprecipitates were prepared from resting and F(ab')2 or RAMIG treated cells and immunoblotted with anti-phosphotyrosine and anti-Gab2 antibodies.

Anti-p85 precipitates were first immunoblotted with anti-phosphotyrosine antibody 4G10. A 1 minute exposure of this blot (Figure 33) showed the rapid tyrosine phosphorylation of a 100 kDa protein, following BCR/FCγRIIB co-ligation, which diminishes gradually and is barely detectable at 15 minutes. This protein was visible at reduced levels after 1 minute following BCR ligation and had almost completely diminished after ten minutes. Examination of a 1 hour exposure of this blot revealed the enhanced tyrosine phosphorylation of a 70kDa band following ligation of the BCR (figure 33). This protein, which was suspected to be SHP2, may represent the phosphorylated p70 protein detected in antiphosphotyrosine blotting of whole cell lysates (figure 31). Additionally, a heavily phosphorylated 62 kDa protein can be seen, and this protein which may represent the p62 protein seen in figure 31. Re-

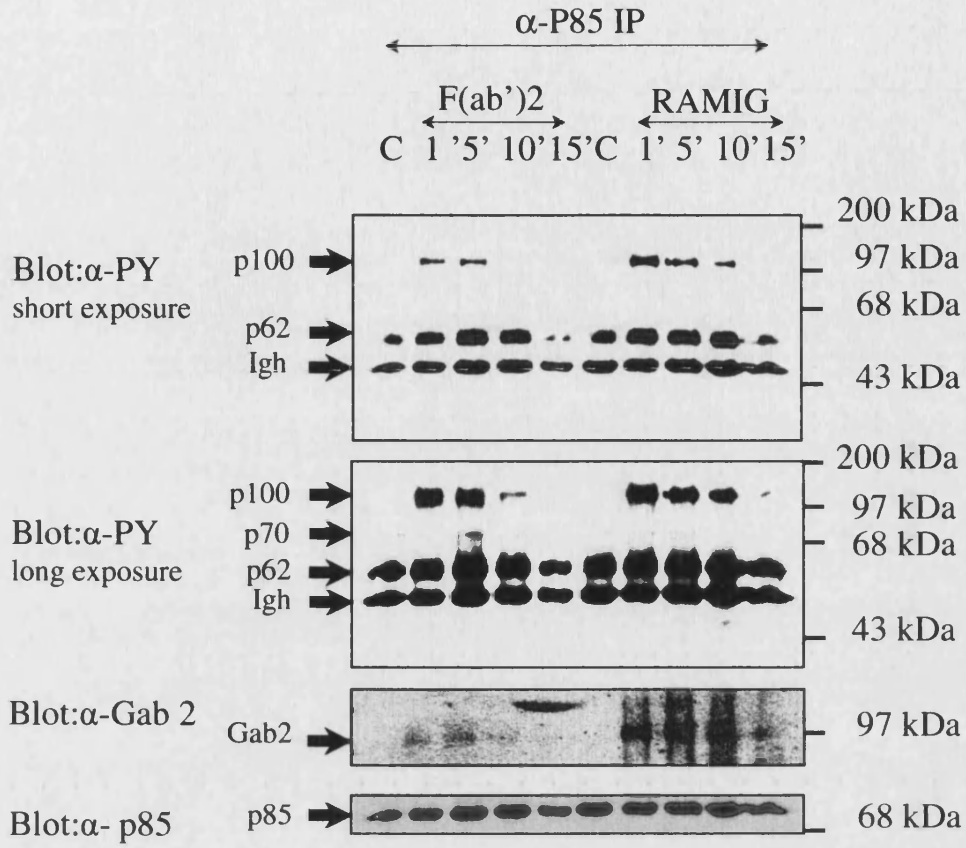


Figure 33: Tyrosine phosphorylation of anti-p85 immuno-precipitates following BCR ligation or BCR/FCγRIIB1 co-ligation.

$2x10^7$ A20 cells were either left unstimulated as a control or stimulated with 20 μg RAMIG or 12 μg F(ab')2 fragments of RAMIG. Cells were lysed and precipitated with 1 μg anti-p85 antibody. Proteins were separated by 7.5 % SDS-PAGE and transferred onto nitro-cellulose. Immunoblotting was carried out with anti phospho-tyrosine antibody 4G10 (top and second panel)at a concentration of 1 μg per ml in 0.05% marvel/PBS, anti-Gab2 (third panel) at 1 μg per ml, and finally with anti-p85 at a concentration of 1 μg per ml, to verify equal loading of immuno-precipitated proteins blots (bottom panel). These data are from a single experiment and are representative of three separate experiments.

probing with anti-Gab2 antibodies (figure 33) readily confirmed that the RAMIG induced p100 band was Gab 2. Overexposure (for 1 hour) (figure 33) was required to detect the lesser quantities of Gab 2 associated with p85 following BCR ligation. Finally re-probing with anti-p85 mAb demonstrated equal loading and transfer of immunoprecipitated protein.

THE N AND C TERMINAL SH2 DOMAINS OF p85 CAN ASSOCIATE WITH GAB2.

Having demonstrated that Gab2 can co-associate with p85 immunoprecipitated complexes in A20 cells, an effort to identify whether this association was based on a direct physical interaction between Gab2 phosphotyrosine residues and p85 SH2 N and C terminal SH2 domains was made. Previously, studies in haemopoietic cells using GST fusion proteins representing the N and C terminal SH2 domains of p85 have demonstrated that Gab2 and p85 can directly interact in response to IL-3 stimulation (Craddock and Welham 1997). This study has employed these constructs which were a kind gift from Mike Waterfield, Ludwig Institute London.

Using these GST fusion proteins, precipitation of proteins from resting or stimulated A20 cells was performed (figure 34). In the first instance, precipitates were immunoblotted with anti-phosphotyrosine antibody 4G10 (figure 34). In both sets of precipitates tyrosine phosphorylated bands migrated at 100 kDa, 70 kDa, and in the GST-p85-NSH2 precipitates alone, at 62 kDa. The 100 kDa protein was the most common precipitated species, and as the aminio acid sequence of the Gab2 suggests, re-probing with anti-Gab2 antibodies demonstrated that this protein was in fact Gab2 (figure 34). The kinetics of the association of Gab2 with both GST-p85-NSH2 and GST-p85-CSH2 proteins, mirrored those previously observed from immunoblotting endogenous p85 immunoprecipitates with 4G10 and Gab2 (figure 34). In short, a greater amount of tyrosine phosphorylated Gab2 protein was seen to co-precipitate with the GST-p85-SH2 fusion proteins at 1 minute following FCγRIIB co-ligation with the BCR, which is decresed at 5 minutes. Meanwhile BCR ligation results in moderate accumulation of tyrosine phosphorylated Gab2 protein in NSH2 immunoprecipitates, which was maximal at 5 minutes.

The association of Gab2 with the GST-p85-CSH2 was reduced in comparison with the association of GST-p85-NSH2 and Gab2 (figure 34). FCγRIIB co-ligation with the BCR induced slightly greater co-association of Gab2 in GST-p85-CSH2

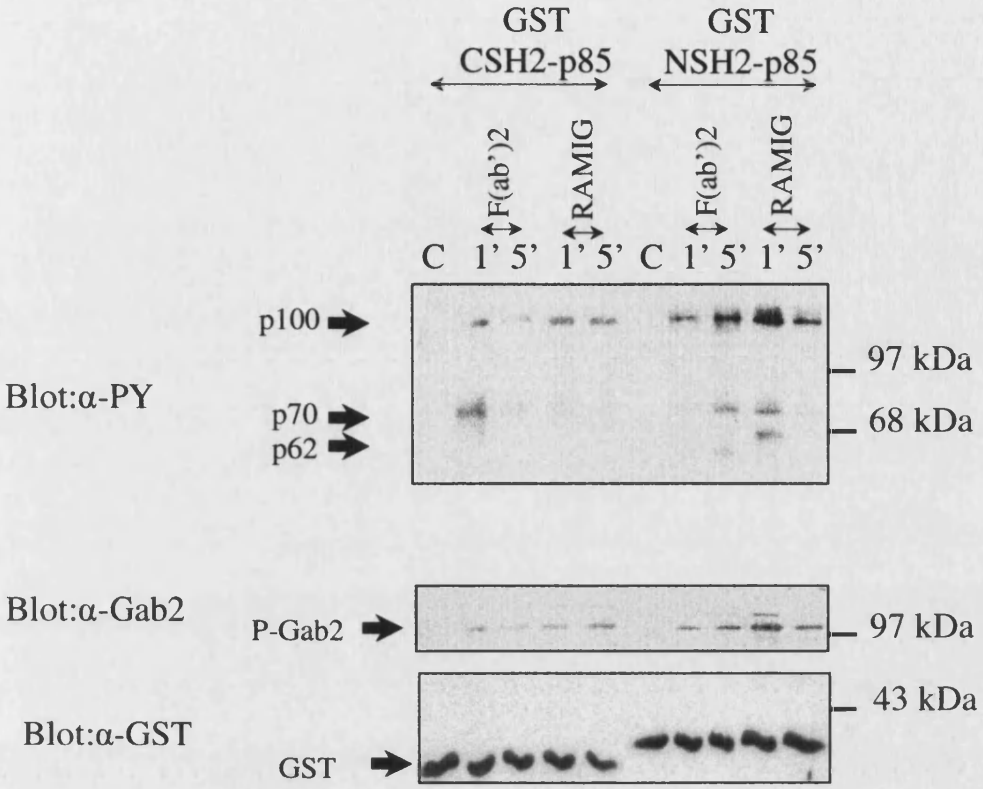


Figure 34: BCR ligation versus BCR/FCγRIIB co-ligation induced tyrosine phosphorylation of N-SH2-p85-GSTor C-SH2-p85-GST precipitated proteins.

$2x10^7$ A20 cells were either left unstimulated as a control or stimulated with 20 μg RAMIG or 12 μg F(ab')2 fragments of RAMIG. Cells were lysed and subject to precipitation with 10 μg N-SH2-p85-GST or 10 μg C-SH2-p85-GST fusion protein. Precipitates were separated using 7-17% gradient SDS-PAGE and transferred onto nitro-cellulose for immunoblotting with anti-phosphotyrosine antibody 4G10 (top panel) at a concentration of 1 μg per ml in 0.05 % marvel/PBS. Blots were subsequently stripped and re-probed with anti-Gab2 (second panel) at 1 μg per ml, and anti-GST at 0.1 μg per ml to verify equal loading of fusion protein (bottom panel). These data are from a single experiment which is representative of four others.

precipitates, and Gab2 levels following both BCR and BCR/FCγRIIB co-ligation peaked at 1 minute and diminished slightly thereafter (figure 34). Furthermore tyrosine phosphorylated proteins were also detected at 70 and 62 kDa (Figure 34). By virtue of their molecular weight, potential candidates for these tyrosine phosphorylated proteins are SHP2 and p62 Dok-1 (figure 34). Re-probing with anti-GST antibody verified equal loading of fusion protein (figure 34).

THE C- AND N- TERMINAL SH2 DOMAINS OF p85 DIRECTLY INTERACT WITH GAB2 FOLLOWING BCR LIGATION AND BCR/FCγRIIB CO-LIGATION IN GAB2 IMMUNOPRECIPITATES.

To confirm that the C- and N- terminal SH2 domains of p85 could interact directly with Gab2, following stimulation through the BCR and BCR /FcγRIIB in A20 cells, Gab2 immunoprecipitates were prepared from resting or stimulated cells and far western blotted with either GST-p85-NSH2 or GST-p85-CSH2 (figure 35). As observed in N-SH2 and C-SH2-p85-GST immunoprecipitates (figure 35), a band of 100kDa was seen (figure 35). Re-probing with anti-Gab2 identified the 100 kDa band as Gab2.

Once more the kinetics of the N-terminal SH2 domain association with Gab2 were strongest following co-ligation of the BCR/FCγRIIB (figure 35) and were maximal after 1 minute. This association increased just above basal levels following BCR ligation alone at 5 minutes. Far-Western blotting with the C-terminal SH2 domain demonstrated similar patterns of co-association, with maximal association between Gab2 and C-SH2 p85-GST occurring following FCγRIIB co-ligation with the BCR, which was elevated at 1 minute (figure 35). This data confirmed that Gab2 forms a direct physical association with p85 via the N and C terminal p85 SH2 domains.

BCR AND BCR/FCγRIIB LIGATION INDUCE TYROSINE PHOSPHO-RYLATION OF SHP2 AND ASSOCIATION WITH MULTIPLE TYROSINE PHOSPHORYLATED PROTEINS.

The presence of a 70 kDa tyrosine phosphorylated band in A20 whole cell lysates and anti-p85 immunoprecipitates was considered to be SHP2 which has been demonstrated to associate with Gab2 and p85 in haemopoietic cells (Craddock and Welham 1997). Furthermore, SHP 2 has been reported to play opposing roles, being implicated in negative signalling in B cells (D'ambrosio et al 1995, Nakamura et al 2000) and positive signalling through the MAP kinase pathway in

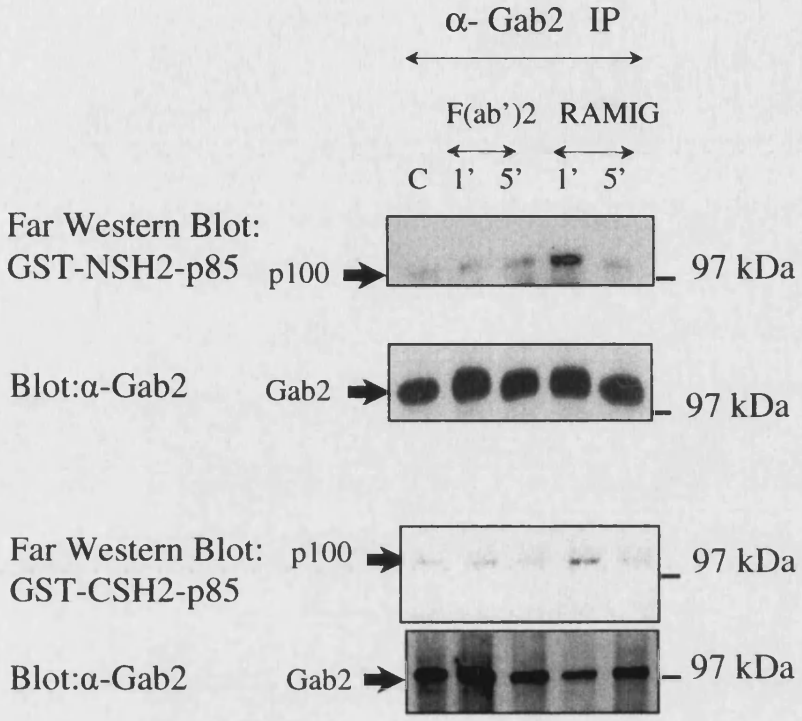


Figure 35: BCR ligation and FCγRIIB co-ligation induced association of a GST protein representing the p85 N and C terminal SH2 domains with a 100 kDa protein Gab2 immuno-precipitates.

2 x 10^7 A20 cells were either left unstimulated or stimulated with 20 μg RAMIG or 12 μg F(ab')2 fragments of RAMIG. Cell lysates were precipitated with anti-Gab2 and separated by 7.5 % SDS-PAGE. Proteins were transferred onto nitro-cellulose and 'far-western' blotted with NSH2 p85-GST (top panel) or CSH2-p85-GST (third panel) at a concentration of o.1 μg per ml in 0.05% Marvel/PBS. Blots were subsequently stripped and re-probed with Gab-2 at 1 μg per ml to verify equal loading and identification of associating proteins (second and bottom panel). These data are from a single experiment and are representative of three separate experiments.

other systems (Noguchi et al 1994, Li et al 1994). Thus it was of interest for the purposes of this study to positively identify this protein and investigate its interaction with Gab2 and p85 in response to FCγRIIB and BCR initiated signals. In the first instance anti-SHP2 immunoprecipitates were derived from BCR and BCR FCγRIIB stimulated A20 cells. Immunoprecipitates were blotted with anti-phosphotyrosine antibody to identify patterns of tyrosine phosphorylation under these conditions. This revealed (figure 36), a considerable level of associating tyrosine phosphorylated proteins and in particular a constitutively phosphorylated band of approximately 62 kD. This band was putatively considered to be p62 DOK and further investigations to assess this possibility were subsequently carried out.

A heavily tyrosine phosphorylated protein, which migrated at around 70 kDa (figure 36 top panel) was thought to be SHP2 and re-probing with anti-SHP2 mAb confirmed this. This band appeared to be tyrosine phosphorylated in response to BCR ligation, which was sustained until 10 minutes post ligation. Meanwhile, FCγRIIB induced a much more transient tyrosine phosphorylation of this protein, which had diminished to almost basal levels by five minutes post-ligation (figure 36). This suggested that SHP2 could be transiently phosphorylated above basal levels by stimulation of the inhibitory pathway in A20 cells, whilst BCR ligation induced a more sustained level of SHP2 tyrosine phosphorylation.

In addition to the phosphorylation of SHP2 and potentially p62, figure 36 shows the tyrosine phosphorylation of a 100 kDa band which re-probing (figure 36) demonstrated to be Gab 2. The association of Gab2 with SHP2 immunoprecipitates following BCR ligation was maximal at five minutes and diminished thereafter. Following FCγRIIB co-ligation with the BCR, a rapid and transient association of Gab2 is detected in anti- SHP2 precipitates which is barely detectable at five minutes post ligation. Re-probing with anti-SHP2 antibody demonstrated the equal loading and transfer of immunoprecipitated protein.

SHP2 GAB2 AND p85 CAN BE DETECTED IN THE SAME COMPLEX IN ANTI-SHP2 PRECIPITATES IN RESPONSE TO BCR/FCγRIIB CO-LIGATION.

SHP2 has been previously described to co-precipitate with p85 (Welham et al 1994), via a p100 protein, now identified as Gab2 (Nishida et al 1999, Craddock and Welham 1997, Gu et al 1998). Following the detection of a p70 kDa band in p85

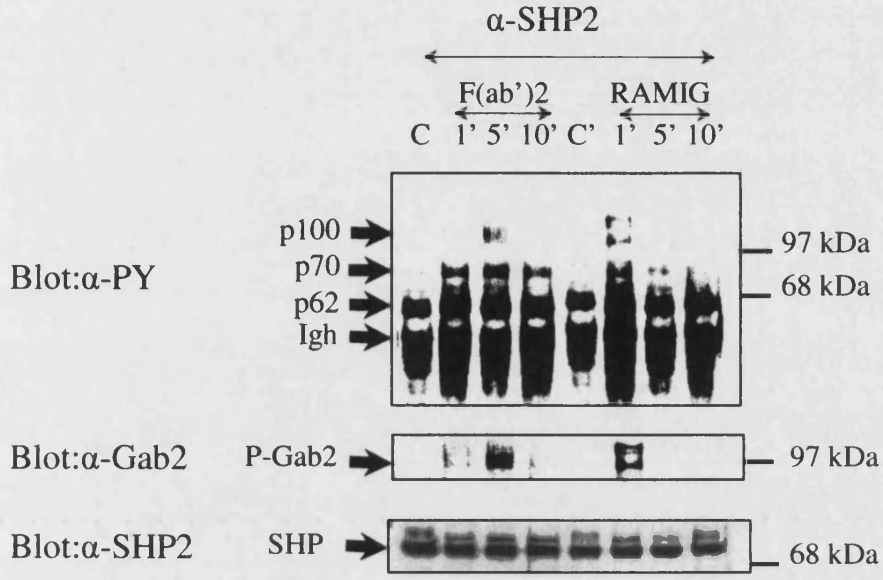


Figure 36: BCR ligation, and FCγRIIB co-ligation, induced tyrosine phosphorylation of anti-SHP2 immunoprecipitated proteins.

2 x10 ^{7} A20 cells were either left unstimulated as a control or stimulated with 20 μg RAMIG or 12 μg F(ab')2 of RAMIG. Cell lysates were immuno-precipitated with 1 μg anti-SHP2 and proteins separated by 7-17% SDS-PAGE. Precipitates were transferred onto nitro-cellulose and probed with anti-phosphotyrosine antibody 4G10 (top panel) at 1 μg per ml, anti-Gab2 (second panel) at 1 μg per ml, and finally anti-SHP2 (bottom panel) at 1 μg per ml to verify equal loading of immunoprecipitated protein. These data are from a single experiment and are representative of three separate experiments.

immunoprecipitates, and the observation that tyrosine phosphorylated Gab 2 can co-associate with SHP2 and p85 precipitated proteins, it seemed important to establish whether p85, could be associating via Gab2 with SHP2. Consequently, anti-SHP2 immunoprecipitates were subject to immunoblot analysis with anti-p85 antibodies. Rapid and sustained co-association of p85 was detected in SHP2 immunoprecipitates following BCR ligation (Figure 37). Similarly following FCγRIIB co-ligation with the BCR p85 was detected in SHP2 immunoprecipitates after 1 minute, but this had dramatically diminished by 5 minutes. Equal loading of SHP2 protein was verified by stripping and re-probing with anti-SHP2 (figure 37).

ASSOCIATION OF GAB 2 WITH GST FUSION PROTEINS REPRESENTING N TERMINAL SH2 DOMAIN, C TERMINAL SH2 DOMAIN AND FULL LENGTH SHP2.

SHP2 and Gab 2 have been reported by this study to co-associate. To define the nature of their physical interaction further, GST fusion proteins expressing the N and C terminal SH2 domains of SHP2 and full length SHP2, were used to precipitate protein from resting or BCR and BCR/FCγRIIB stimulated A20 cells. Immunoblotting of precipitates with anti-Gab2 identified the kinetics and strength of Gab2 association with each SH2 domain and compared each of these to the association of Gab2 with full length SHP2.

Immunoblotting of full length GST-SHP2-SH2 precipitates revealed that the association between GST-SHP2-SH2 and Gab2 is greatest following co-ligation of the BCR/FCγRIIB (figure 38). Co-association was greatest at one minute post co-ligation and rapidly diminished from this point. BCR ligation lead to a gradual accumulation of Gab2 which peaked at ten minutes post stimulation. Conversely, C-terminal SHP2-SH2 precipitates blotted with Gab2 (figure 38), revealed that the greatest association between Gab2 and the C-terminal SH2 domain of SHP2 occurred following BCR ligation alone, with the greatest association detected at one minute. However, considerable co-association could also be detected at 5 minutes following BCR/FCγRIIB co-ligation. Finally, the lower panel of figure 38 demonstrates the association of Gab 2 with the N- terminal SH2 domain of SHP2. Here, as with the full length SHP2-SH2 immunoprecipitates the N terminal SH2 domain can be seen to associate at far greater intensity with Gab 2 following BCR/ FCγRIIB co-ligation.

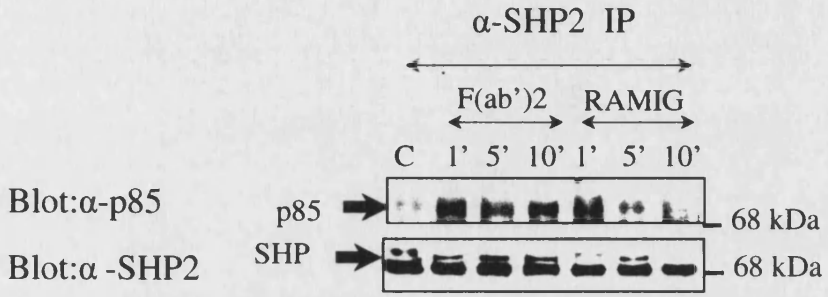


Figure 37: BCR ligation versus BCR/FCγRIIB1 co-ligation induced association of Gab2 and p85 with anti-SHP2 precipitated proteins.

$2x10^7$ A20 cells were either left unstimulated or stimulated with 20 μg RAMIG or 12 μg Fab'2 fragments of RAMIG. Cells were lysed and proteins were subsequently immunoprecipitated using 1 μg anti-SHP2 rabbit polyclonal antibody. Immune-complexes were separated via 7.5% SDS-PAGE, transferred onto nitro cellulose and sequentially immunoblotted with anti- Gab2 antibody (top panel) at a concentration of 1 μg per ml in 0.05 % marvel/PBS , anti- p85 (second panel) at 1 μg per ml, and finally SHP2 (bottom panel) at 1 μg per ml to confirm equal loading of immunoprecipitated proteins. Blots were stripped before each reprobe. These data are from a single experiment which was representative of two separate experiments.

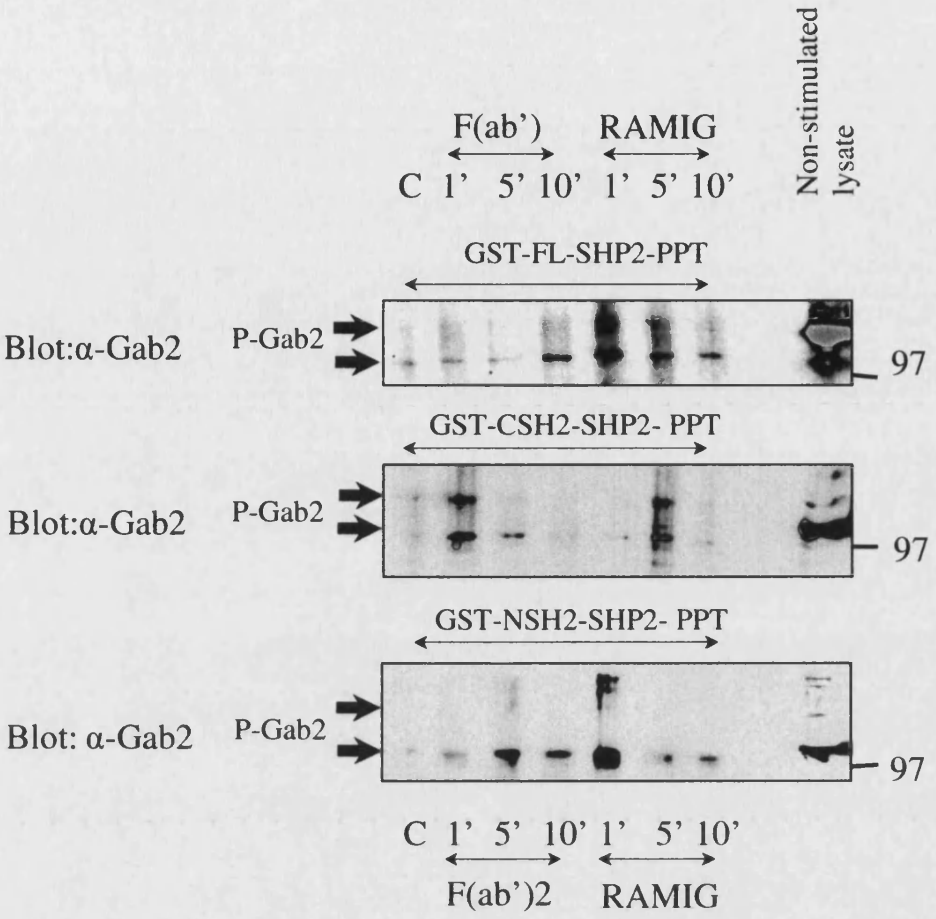


Figure 38: BCR ligation versus BCR/FCγRIIB1 co-ligation stimulated association of Gab2 with SHP2 N-terminal SH2 domain, C-terminal SH2 domain, and full length (FL) GST fusion proteins.

A20 cells were either left unstimulated as a control or stimulated with 20 μg RAMIG or 12 μg F(ab')2 RAMIG for the time points indicated. 20x 10^6 cells per point were lysed and proteins were immunoprecipitated with GST fusion proteins and glutathione sepharose. Precipitated proteins were separated via 7-17% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with anti-Gab2 at 1μg per ml antibody in 0.05% marvel/PBS (all panels). Proteins were visualised by chemiluminescence. These data are from a single experiment, the C-SH2 and FL-SH2 SHP2 -GST IPS are representative of two experiments, whilst the NSH2-SHP2-GSTIPS are representative of four separate experiments.

SUMMARY

1) Elevated levels of *in vitro* lipid kinase activity can be detected in association with anti- phosphotyrosine immunoprecipitates derived from BCR/FCγRIIB co-ligated A20 cells, as compared to immunoprecipitates from cells stimulated via the BCR alone.
2) BCR ligation induces the tyrosine phosphorylation of proteins at 145 kDa 100kDa and 70 kDa. The tyrosine phosphorylation of p145 and p100 is enhanced in response to BCR/FCγRIIB co-ligation whilst phosphorylation of p70 is reduced.
3) The p100 phospho-protein in A20 whole cell lysates was identified as Gab2 and is subject to rapid and considerable tyrosine phosphorylation in response to BCR/FCγRIIB co-ligation. BCR ligation induces more gradual, and moderate tyrosine phosphorylation of Gab2.
4) Tyrosine phosphorylated Gab2 co-associates with the regulatory subunit of PI3K, p85 and this association is enhanced following BCR/ FCγRIIB co-ligation.
5) Gab2 directly co-associates most strongly with p85 via the N terminal SH2 domains of p85 following BCR/ FCγRIIB co-ligation. Weak association with the p85 C terminal SH2 domain can also be observed and is also greatest under inhibitory signalling conditions.
6) Tyrosine phosphorylation of SHP2 is transiently induced in response to BCR/FCγRIIB co-ligation, and diminishes after 1 minute. In contrast BCR ligation induces the gradual tyrosine phosphorylation of SHP2 which is sustained at ten minutes.
7) SHP2 co-precipitates with Gab2. This co-association is rapid and transient following FCγRIIB co-ligation and is undetectable after 1 minute. Furthermore this association appears to be mediated predominantly by the N terminal SH2 domain of SHP2 under negative signalling conditions, whilst following BCR ligation, the C terminal SH2 domain can interact with Gab2.
8) p85 is also detected in SHP2 immunoprecipitates, with kinetics which mirror the phosphorylation and co-association of SHP2, and association with Gab2.

3.3 DISCUSSION

3.3.1 FCγRIIB MODULATION OF THE FUNCTIONAL READOUTS OF BCR MEDIATED SIGNALLING CASCADES IN A20 CELLS.

The mobilisation of calcium, observed in this study to occur upon BCR ligation correlates with data presented by previous studies, which demonstrated that BCR ligation induces a massive calcium influx in B cells (Coggeshall *et al* 1991, Carter *et al* 1991). Studies which have examined the intracellular events that mediate this response demonstrate that the BCR stimulates the assembly of PLCγ-BTK-BLNK (Takata *et al* 1996, Ishai *et al* 1999) complexes. In this way PLCγ activation is induced through proximity with the Tec kinase Btk, and the ensuing accumulation of inositol polyphosphates initiates the mobilisation of intracellular calcium (Buhl *et al* 1998, Lankester *et al* 1996, Li *et al* 1998, Nagai *et al* 1995). Furthermore, this study has described the effective inhibition of calcium mobilisation that can be achieved via RAMIG mediated co-ligation of the FCγRIIB with the BCR. This is also in accordance with previous studies which have shown that co-ligation of the ITIM bearing inhibitory receptor, FCγRIIB, with the BCR leads to the inhibition of calcium mobilisation (Diegel *et al* 1994). The SHIP mediated enzymatic degradation of PIP_3 which prevents the activation of Btk, and its subsequent phosphorylation of PLCγ is proposed to be the major determining factor in the abrogation of calcium signalling (Ono *et al* 1997).

This study also demonstrates that ERK1/2 MAP kinases could be phosphorylated in a BCR dependent manner and that this phosphorylation was abrogated by FCγRIIB co-ligation with the BCR. These observations indicated that positive and negative signalling cascades in the A20 B cell line could be optimally stimulated with regard to ERK activation. These data are in accordance with previous work by other researchers, which has shown that ERK activation can be achieved following BCR ligation. BCR/CD19 proximal events include the association of Vav with CD19 (Gulbins *et al* 1997), the phosphorylation of Shc the association of Shc with the Igα chains of the BCR (Lankester *et al* 1996) and the assembly of Shc/Grb 2/SOS complexes (Nagai *et al* 1995). The observed association of Vav with CD19 (Gulbins *et al* 1997), and the accumulation of Grb2/SOS complexes in response to BCR triggering (Harmer *et al* 1993), mediate phosphorylation of ERK1/2/MAP kinases (Li *et al* 1998). FCγRIIB mediated inhibitory signals lead to the depressed activation of the MAP kinase pathway (Sarmay *et al* 1996, Moodie *et al* 1994, Campbell *et al* 1995).

This study has demonstrated that the efficient coupling of BCR mediated pathways, which lead to the activation of PI3K, can be achieved by the treatment of A20 cells with F(ab')2, and can be abrogated following co-ligation of the BCR with the FCγRIIB receptor, by treatment of cells with RAMIG. In accordance with previous studies (Tuveson *et al* 1997, Gold *et al* 1994) the recruitment of p85 to the CD19 receptor is observed in (Fab')2 treated A20 cells, and is accompanied by a marked accumulation of PI(3,4,5)P_3. The BCR mediated upregulation of PI3K is essential for the downstream activation of PH domain containing proteins PKB (Gold *et al* 1999, Astoul *et al* 1999) and Btk (Buhl *et al* 1999), which, respectively, play important roles in enhancing cell survival (Coffer *et al* 1998) and mediating calcium mobilisation (Fluckiger *et al* 1997).

3.3.2 REGULATION OF PI(3,4,5)P_3 ACCUMULATION BY SHIP

FCγRIIB mediated inhibition of PI(3,4,5)P_3 accumulation has been observed by this and previous studies (Scharenberg *et al* 1998), following the treatment of A20 cells with RAMIG. The inhibited accumulation of this lipid could be attributable to the dephosphorylation of CD19, that has been previously described (Kiener *et al* 1997) to occur upon FCγRIIB co-ligation, and which prevents the co-association and activation of PI3K via CD19. The absence of p85 protein, detected in this study upon immunoblotting of anti-CD19 immunoprecipitates, reflects the abrogation of p85 recruitment to CD19, and may explain the observed reduction in PI(3,4,5)P_3 accumulation. Furthermore, the enzymatic activation of the 5'-inositol poly phosphatase SHIP by the FCγRIB has been described as a major effector of the inhibitory signal through the dephosphorylation of PI(3,4,5)P_3 (Ono *et al* 1996 and Bolland *et al* 1998). This study has demonstrated the FCγRIIB mediated tyrosine phosphorylation of SHIP. Tyrosine phosphorylation has been observed to correlate with catalytic activation of SHIP in T cells (Edmunds *et al* 1999), which suggests that the accumulation of PI(3,4,5)P_3 in A20 cells may be regulated both by the dephosphorylation of CD19 and the enzymatic activation of SHIP.

The enzymatic regulation of PI(3,4,5)P_3 levels by SHIP would result in the accumulation of SHIP's metabolic product PI(3,4)P_2. However measurement of PI(3,4)P_2 accumulation by this study demonstrated that reduced levels of this lipid accumulate following FCγRIIB co-ligation, as compared to ligation of the BCR alone. These data could indicate that SHIP does not mediate a catalytic role in inhibitory

signalling, which would suggest that the diminished level of PI(3,4,5)P_3, observed following FCγRIIB coligation, is a result of the non-phosphorylation of CD19. If this were the case then the essential role that has been described for SHIP, in mediating the FCγRIIB signal (Scharenberg *et al* 1996), must be elicited by SHIP's structural motifs which lie outside its phosphatase domain.

The presence of multiple sites for protein-protein interaction within the SHIP protein have previously implicated SHIP in mediating an adaptor function in inhibitory signalling. Interestingly one study has described the co-precipitation of p85 by tyrosine phosphorylated SHIP, via the SH2 domains of p85 (Gupta *et al* 1998). Thus SHIP may prevent the activation of PI3K via the sequestration of p85 away from active signalling complexes. However no evidence for this role could be provided here as co-association between SHIP and p85 could not be detected by this study. Support for an adaptor function for SHIP can be gained from previous work which has examined conserved motifs within the SHIP protein. The C terminal region of SHIP contains two key tyrosine residues which have been previously described to mediate associations with Shc in T cells (Lamkin *et al* 1997) and B cells (Tridandapani *et al* 1997) and p62 Dok in B cells (Tamir *et al* 2000). The SH2 domain of non-phosphorylated SHIP preferentially mediates SHIP's interaction with the FCγRIIB ITIM (Muta *et al* 1994) whilst the SH2 domain of tyrosine phosphorylated SHIP SH2 preferentially binds to the Y^{317} residue of Shc (Tamir *et al* 2000), competing at this site with the Grb2 SH2 domain, for binding to Shc (Tridandapani *et al* 1997). Furthermore recent studies have demonstrated that truncation of the C terminal 190 amino acids of SHIP impairs SHIP's ability to inhibit calcium mobilisation (Aman *et al* 2000). The constitutive membrane localisation of truncated SHIP can restore SHIP's inhibitory function, and thus it has been proposed that this region may regulate SHIP's inhibitory function via the membrane localisation of SHIP (Aman *et al* 2000).

There is therefore much evidence to support a role for SHIP in protein-protein interactions that contribute to the FCγRIIB inhibitory cascade. However, further studies have equally demonstrated a role for SHIP's phosphatase activity. Membrane expression of catalytic mutants of SHIP in B cells inhibited FCγRIIB elicited membrane recruitment of Btk (Bolland *et al* 1998) and calcium mobilisation (Ono *et al* 1996). Furthermore it was recently observed that a 135 kDa isoform of SHIP, which is

catalytically active but cannot interact C terminally with PI3K and lacks proline rich regions that are predicted to interact with SH3 containing molecules, is sufficient to mediate inhibitory signalling in B cells (March *et al* 2000).

Thus the enzymatic activity of SHIP cannot be disregarded when interpreting the findings of this current study. It could be considered, therefore that the lack of FCγRIIB mediated PI(3,4)P_2 accumulation observed here was due to the removal of this lipid, or its progenitor PI(3,4,5)P_3, by lipid phosphatases with specificity for phosphoinositdes phosphorylated at positions other the 5'-OH on the inositol ring. In accordance with this possibility, the low level expression of the tumour suppressor 3' phosphatase, PTEN, was detected in A20 cells and was described earlier in this study. The 3'-phosphatase activity of PTEN would negatively regulate the accumulation of PI(3,4)P_2, or PI(3,4,5)P_3 and would result in the accumulation of PI(4)P or PI(4,5)P_2 respectively. Thus the concerted efforts of SHIP and PTEN may regulate 3'-phosphoinositide levels following FCγRIIB co-ligation with the BCR, and it would be interesting to assess this relationship in future studies.

This study demonstrated the reduced yet moderate phosphorylation of SHIP following BCR ligation alone. Although the rapid tyrosine phosphorylation of SHIP, in response to FCγRIIB coligation with the BCR is widely accepted, Ship's tyrosine phosphorylation in response to BCR ligation is described in previous studies to occur to varying extents (Sarkar *et al* 1995, Chacko *et al* 1996). Thus SHIP may be active in BCR mediated pathways, which may explain the elevated accumulation of PI(3,4,5)P_3, and mobilisation of intracellular calcium, that is observed upon BCR ligation of SHIP-/-B cells (Bolland *et al* 1998).

To conclude, It appears that the inhibitory signal mediated by the FCγRIIB receptor in A20 cells mediates the abrogation of PI3K dependent signalling pathways either via the prevention of PI3K activation, by lack of CD19 phosphorylation, or through the enzymatic activation of SHIP. In addition the actions of other lipid phosphatases e.g. PTEN, may be involved in regulating PI3K dependent pathways in B cells. Furthermore the abrogation of non-PI3K dependent pathways which contribute to MAPK activation may also be brought about by SHIP, through its ability to act as an adaptor protein.

3.3.3 REGULATION OF PI3K BY ITS RECRUITMENT TO TYROSINE PHOSPHORYLATED PROTEIN COMPLEXES

Work presented by this study has examined the hypothesis that BCR and FCγRIIB mediated signalling cascades differentially regulate the association of PI-3K activity with tyrosine phosphorylated complexes *in vitro*. Data suggests, that in intact cells, the global production of $PI(3,4,5)P_3$ is suppressed under inhibitory signalling conditions. Hence, these *in vitro* observations in response to FCγRIIB co-ligation, may represent either a) the sequestration of lipid kinase activity in phosphotyrosyl complexes, that may direct PI3Ks' approximation with SHIP; or b) the elevated association of PI-3K protein in complexes that prevent its activation by competing for the p85 SH2 domains, that under positive signalling conditions mediate PI3K activation through membrane localisation via co-association with cell surface receptors.

In addition to a role for SHIP in mediating inhibitory signalling cascades through its' secondary function as an adaptor protein, other adaptor proteins may mediate the suppression of PI3K dependent signalling, through the sequestration of the p85 regulatory sub-unit. Examination of lipid kinase activity associated with anti-phosphotyrosine immunoprecipitates suggested that an association of PI3K with tyrosine phosphorylated complexes under negative signalling conditions, may exist. It is unclear whether the increased lipid kinase activity observed following FCγRIIB coligation is a direct result of upregulation of a pool of PI3K that is associated with tyrosine phosphorylated proteins, or an enhanced recruitment of basally active PI3K protein into phosphotyrosine complexes. If the former is the case then, to achieve the overall reduction in PIP_3 accumulation observed in response to FCγRIIB co-ligation in intact cells, the FCγRIIB enhanced PI3K activity might be redistributed into the vicinity of enzymatically active SHIP, via tyrosine phosphorylated complexes. A similar analogy was put forward by Gupta *et al* (1998), in that they observed the sequestration of p85 via a direct interaction between the C terminal SH2 domain of p85 and tyrosine phosphorylated SHIP. It was postulated that under negative signalling conditions SHIP is responsible for the activation of PI3K **and** the degradation of its' lipid products to PIP_2 (Gupta *et al* 1998). It was proposed that whilst the degradation of $PI(3,4,5)P_3$ by SHIP abrogates the activation of Btk, the accompanying production of $PI(3,4)P_2$ could be responsible for the residual activation of PKB (Gupta *et al* 1998). If argument b) stated above were the case then the recruitment of PI3K to tyrosine phosphorylated protein complexes may serve to sequester inactive PI3K and prevent its activation. The

identity of alternative phosphotyrosine containing proteins, that could mediate the sequestration of p85 and/or direct its approximation with SHIP, has therefore been examined by this study.

3.3.4 GAB2 AND SHP2 IN FCγRIIB MEDIATED MODULATION OF PI3K AND MAPK ACTIVATION

The demonstration that the 100 kDa scaffold protein, Gab2, was heavily phosphorylated upon FCγRIIB co-ligation with the BCR, suggested that this protein may be implicated in negative signalling in B cells. Previous work has repeatedly demonstrated the co-association of a 100 kDa protein with p85 via the p85 SH2 domains (Craddock and Welham 1997, Gadina *et al* 1999) and recent cloning designated this protein as Gab2 (Nishida *et al* 1999). Gab2 has been implicated in mediating positive signalling linking receptors to ERK activation (Nishida *et al* 1999). In a more recent study, Gab2's coassociation with p85 has been shown to be essential for activation of PI3K and IL-3 dependent activation of the AKT pathway (Gu *et al* 2000). In the same study Shc was implicated in mediating tyrosine phosphorylation of Gab2 and initiating a shc/grb2/Gab2/PI3K/AKT pathway in response to IL-2 receptor signalling (Gu *et al* 2000). A role for Gab2 in mediating positive signals in T cells has also been described by studies which have shown that IL-2 and IL-15 receptor stimulation can couple to MAPK activation via Jak3 elicited tyrosine phosphorylation of Gab2 (Gadina *et al* 2000). Interestingly, the same study demonstrated that expression of Gab2 protein is upregulated upon T cell activation, further implicating this protein in the delivery of upstream signals to downstream components of lymphocyte function (Gadina *et al* 2000). However, further studies have indicated an inhibitory role for Gab2 in uncoupling signals from ERK to transcriptional activation of ELK-1 triggered by a dominant active Ras mutant (RasV12) or under IL-3 stimulation (Zhao *et al* 1999).

This study provides the first demonstration that Gab2 is a substrate for PTK activity mediated upon BCR/FCγRIIB co-ligation. Furthermore it has been shown here that tyrosine phosphorylation of Gab2 is reduced upon BCR ligation as compared to that induced by BCR/FCγRIIB co-ligation. This study also provides novel data concerning the enhanced co-association of Gab2 with p85 in response to BCR/FCγRIIB mediated signals. Thus, a close correlation between the association of p85 with Gab2, and the tyrosine phosphorylation of Gab2, exists. This observation is in accordance with prior studies which observed that the co-association of Gab2 and p85 is mediated by the SH2

domains of p85 upon cytokine receptor stimulation (Craddock and Welham 1997), which can interact with tyrosine phosphorylated YXXM motifs in the Gab2 amino acid sequence (Nishida *et al* 1999). This study demonstrated that co-association of Gab2 and p85 can occur in A20 cells in response to BCR mediated signals, and that this association is elevated following FCγRIIB co-ligation. Furthermore this interaction was mediated most strongly via the N terminal SH2 domain of p85. The direct physical nature of this interaction was supported by far western blotting with GST p85 N and C SH2 fusion proteins.

This study has also examined the role of SHP2 in B cell receptor and FCγRIIB mediated signalling cascades. The protein tyrosine phosphatase SHP2 has been previously implicated in negative signalling in B cells, through its association, upon FCγRIIB/BCR coligation, with the phosphorylated ITIM (D'ambrosio *et al* 1995). SHP2 binds the ITIM with the second greatest affinity after SHIP, and before SHP1 (previous reference). Studies which blocked the association of SHP2 with the ITIM have concluded that SHP2 plays a minimal role (Nakamura *et al* 2000) in suppressing ERK activation and calcium mobilisation following FCγRIIB co-ligation. Conversely in growth factor mediated signalling cascades SHP2 has been implicated in pathways leading to cell cycle progression (Bennet *et al* 1996), cell survival (Pazdrak *et al* 1997) and mitogenesis (Xiao *et al* 1994). Thus, the biochemical nature of SHP2's role following FCγRIIB co-ligation with the BCR, in B cells is poorly understood. A single study has reported a co- association between SHP2 with a 120-kDa protein which occurs upon BCR ligation in RAMOS B cells, and is reduced following FCγRIIB co-ligation with the BCR (Nakamura *et al* 1998). This protein was later putatively identified as Gab1 (Nishida *et al* 1999), which has been implicated in the activation of ERK1/2 MAP kinases (Takahashi Tezuka *et al* 1998).

This study has presented novel data which demonstrates that tyrosine phosphorylation of SHP2 is mediated in response to BCR ligation and BCR/FCγRIIB co-ligation in A20 cells. Previous studies have linked tyrosine phosphorylation of SHP2 in other cell types to MAPK activation (Bennett *et al* 1994): PDGF receptor mediated signalling pathways elicit the phosphorylation of the SHP2 C terminal tyrosine motifs which allow the association of SHP2 with the Grb2 SH2 domain. Subsequent association of the Grb2 SH3 domain with SOS has been shown to link SHP2 to the activation of Ras, and hence to the initiation of the pathways leading to MAPK activation (Bennett *et al* 1994).

Thus, SHP2 phosphorylation, observed by this study following BCR ligation, could implicate SHP2 in coupling the BCR to ERK 1/2 phosphorylation (see Diagram 4). BCR ligation has already been described to couple to MAPK signalling pathways via Vav (Gulbins *et al* 1997) and the assembly of Shc/Grb 2 complexes (Nagai *et al* 1995). This study may describe a further route via which SHP2 may couple B cell receptor stimulation to MAPK signalling pathways.

Following FCγRIIB co-ligation, the phosphorylation of SHP2 is rapidly attenuated . Such de-phosphorylation would abrogate any SHP2 association with Grb2, and remove this route to MAPK activation. The mechanism of de-phosphorylation of SHP2 is unclear. The reported activation of the PTPase activity of SHP1 under negative signalling conditions (D'ambrosio *et al* 1995) could mediate the de-phosphorylation of SHP2. If this were the case SHP1's known associations with Grb2 and mSOS in haemopoietic cells (Kon-Kozlowski *et al* 1996), could act to bring SHP1 into proximity with SHP2, where it could elicit the de-phosphorylation of SHP2, and abrogate any SHP2 phosphotyrosine based molecular interactions.

The assignment of a potential role for SHP2 in BCR/FCγRIIB mediated pathways leading to ERK1/2 activation/ abrogation may add to our understanding of SHP2's role in B cells. However, the association of SHP2 with the FCγRIIB ITIM, and the role that this interaction plays in inhibitory signalling cascades is still not clear. Previously an interaction between SHP2 and Gab2 has been demonstrated and Gab2 has been described as a potential substrate for SHP2 catalytic activity (Craddock and Welham 1997, Zhang *et al* 1998, Yamauchi *et al* 1995). Further examination of the tyrosine phosphorylated proteins immuno-precipitated with SHP2 by this study has led to the identification of an association between Gab2 and SHP2. In the context of insulin and EGF mediated signalling pathways, the catalytic activity of SHP2 is essential for linking receptor stimulation to Raf1 (Milarski *et al* 1994, Bennett *et al* 1996), and interestingly Gab2 has been implicated in coupling the enzymatic activity of SHP2 to ERK, as overexpression of Gab2 leads to the enhanced phosphorylation of ERK (Nishida *et al* 1999). However inhibition of SHP2 /Gab2 interactions via the mutation of the SHP2-SH2 domain binding motifs of Gab2 leads to enhanced MAPK activation. Thus the mechanism whereby the Gab2/SHP2 interaction couples to ERK MAPK activation is not straightforward. It was therefore considered what role may be played by the Gab2

SHP2 interaction in A20 cells, and whether the enzymatic activity of SHP2 was involved.

In accordance with previous studies (Craddock and Welham 1997, Gadina *et al* 1999, Zhang *et al* 1998) Gab2's association with SHP2, measured in this study is closely correlated with the tyrosine phosphorylation of the associated Gab2 protein. This suggests that the association is mediated via Gab2 phosphotyrosine interaction with SH2 domains of SHP2. Observation of a larger band shift of the Gab2 protein was observed following FCγRIIB coligation than following BCR ligation alone (N.B. the enhanced resolution of the p 100 band shift seen here was visualised when precipitates were run on 7-17% gradient gels). Further examination of Gab2 proteins precipitating with GST fusion proteins representing SH2 domains of SHP2 demonstrates that the lower levels of Gab2 phosphorylation induced by BCR ligation are sufficient to mediate an interaction with the C terminal SHP2 SH2 domain. However upon BCR/FCγRIIB co-ligation, enhanced levels of Gab2 phosphorylation are sufficient to mediate a stronger interaction between Gab2 and both SHP2 SH2 domains.

Data presented here demonstrates that the kinetics that characterise the Gab2 and SHP2 interaction, following BCR ligation or FCγRIIB co-ligation are distinct. The ligation of the BCR induces a steady accumulation of Gab2 in SHP2 immunoprecipitates, which diminishes after five minutes. Contrastedly, FCγRIIB co-ligation mediates the rapid and transient co-association of Gab2 with SHP2, which is completely abrogated after 1 minute. The abrogation of the presence of Gab2 in SHP2 immunoprecipitates following FCγRIIB/ BCR co-ligation may represent the enzymatic activation of SHP2, following the occupancy of both N and C terminal SH2 domains, an event which has been proposed to activate SHP2 PTP activity (Pluskey et 1997). Activation of SHP2 PTP activity, would lead to a reduction in the tyrosine phosphorylation of its substrates and thus abrogate SHP2-SH2 domain/ substrate complexes. The FCγRIIB mediated abrogation of Gab2 in SHP2 immunoprecipitated complexes, which is preceded by the interaction of the N and C terminal SH2 domains of SHP2 with Gab2, may allude to a situation where Gab2 can act as a substrate for PTP activity of SHP2 in B cells.

Previous reports have indicated that p85 and SHP2 may exist in the same complex (Welham *et al* 1994, Craddock and Welham 1997, Zhang *et al* 1998) and this study

observed the association of SHP2 with anti-p85 immunoprecipitates. Due to the lack of any known p85 binding motifs within SHP2 previous studies have suggested that SHP2 may couple to p85 via an indirect route, and proposed that the p100 protein seen also in association with SHP2, and p85 immunoprecipitates may mediate this interaction (Craddock and Welham 1997). The similarity in the kinetics of p85/SHP2 co-association and SHP2/Gab2 co-association observed by this study in response to BCR ligation and FCγRIIB co-ligation, is in accordance with a role for Gab2 in mediating the association between p85 and SHP2. Furthermore the presence of a 70 kDa protein in GST-NSH2-p85 precipitates at early time points demonstrates that the co-precipitation of SHP2 with p85 is mediated via the p85 SH2 domains and this may further indicate a trimeric complex in which Gab2 is the intermediate protein.

The following model could be put forward to describe the role of SHP2 and Gab2 in B cells: Under positive signalling conditions SHP2, binds, via its C terminal SH2 domain, to minimally phosphorylated Gab2. BCR ligation also imparts the tyrosine phosphorylation of SHP2 which could facilitate the binding of Grb2 via its SH2 domain to the C-terminal phosphotyrosine motif of SHP2. SHP2 could thus mediate the recruitment of Grb2-SOS complexes and allow the subsequent activation of Ras, providing an additional pathway via which the BCR could couple to Map Kinase activation. The presence of low levels of Gab2 coupled to SHP2 via the SHP2 C terminal SH2 domain could stabilise the SHP2, Grb2 complex, through binding via the Gab2 proline rich motif to Grb2's SH3 domain, in the manner described for Shc, Grb2, SOS complexes (Harmer *et al* 1999). As Grb2/Gab2 associations do not occupy the N-terminal SH3 domain of Grb2 (Zhao *et al* 1999), through which Grb2 associates with SOS, this stabilising interaction would allow association and activation of SOS exchange activity. In addition to MAPK activation, BCR induced phosphorylation of CD19 allows the association and activation of PI3K, leading to the accumulation of PI(3,4,5)P_3, and stimulation of PI3K dependent signalling cascades, which lead to PKB activation, BTK activation and calcium mobilisation.

Under negative signalling conditions, initiated by FCγRIIB co-ligation, initial phosphorylation of SHP2 is rapidly abrogated , which causes its dissociation from Grb2. Meanwhile, FCγRIIB activated PTKs elicit the enhanced phosphorylation of Gab2 which then binds both SH2 domains of SHP2. This event mediates the activation of SHP2 PTPase activity, and its subsequent dissociation from Gab2 complexes. Under

negative signalling conditions, therefore, SHP2 is un-coupled from both Gab2 and Grb2, the binding partners through which it has been described to couple to Ras, and Map Kinase activation. FCγRIIB co-ligation also induces the hyper- tyrosine phosphorylation of Gab2, which enhances the association of the N- and C- terminal SH2 domains of p85 with the Gab2 protein. The enhanced association of p85 with Gab2 correlates with the BCR/FCγRIIB co-ligation mediated reduction in p85 association with CD19. A working model is therefore proposed whereby the marked increase in association of Gab2 with the SH2 domains of p85, observed under inhibitory signalling conditions, may represent the sequestration of p85 away from residually phosphorylated CD19.

With regard to the above hypothesis, a very recent paper has described a negative regulatory role for Gab 2 in T cells which leads to inhibition of Il-2 transcription and is dependent on an association between p85 an Gab2 (Pratt *et al* 2000). The inhibitory relationship observed between Gab2 and p85 by Pratt *et al* was dependent on PI3K activity, and it was suggested that PI3K products binding to the PH domain of Gab2 may mediate the localisation of Gab2 /PI3K complexes to a subcellular site where they can mediate the observed negative effect. The PH domain of Gab2 has not been considered by the current study however the initial observation made here that considerable *in vitro* lipid kinase activity can be immunoprecipitated with tyrosine phosphorylated complexes under negative signalling conditions, may reflect the enhanced Gab2 co-association with active PI3K under these conditions.

Finally, SHP2's association with the FCγRIIB ITIM under negative signalling conditions may mediate the approximation of Gab2 with FCγRIIB activated PTKs, and elicit hyper-phosphorylation of Gab2. Gab2 can then bind both SHP2 SH2 domains, abrogating the association of SHP2 with the ITIM, and activating its PTPase activity. The consequent dissociation of SHP2 from Gab2, would cause the release of SHP2 from signalling complexes at the membrane. The PH domain of Gab2 may allow it to remain in the vicinity of the FCγRIIB where it could sequester Grb2 and bring p85 into the vicinity of SHIP. Thus the enzymatic activity of SHP2, which has been described to be essential for ERK activation downstream of positive signalling pathways, may act under negative signalling conditions in B cells to release Gab2 and p85 into the 'inhibitory signalling environment' of the FCγRIIB.

3.3.5 - CONCLUSIONS

This study has examined the mechanisms which regulate PI3K activity in B cells. t has shown that FCγRIIB co-ligation with the BCR stimulates the hyper-phosphorylation of the adaptor molecule Gab2 which correlates with the enhanced association of the p85 regulatory subunit of PI3K, via its N and C terminal SH2 domains, with Gab2. It is proposed that this interaction may reflect the sequestration of PI3K by Gab2 under inhibitory signalling conditions (diagram 17).

Furthermore, the PTPase SHP2 is subject to sustained tyrosine phosphorylation following BCR ligation and can co-associate mainly via its C-terminal SH2 domain with Gab2. It is proposed that this may mediate SHP2 association with Grb2, which has been reported previously to enhance pathways leading to MAPK activation.
FCγRIIB coligation with the BCR mediates the transient tyrosine phosphorylation of SHP2, and elicits the association of N and C terminal SH2 domains of SHP2 with Gab2. It is proposed that this interaction activates SHP2 catalytic activity, which targets Gab2 and releases SHP2 and from the Gab2/p85 protein complex, which serve to prevent SHP2 mediated MAPK activation (diagram 18).

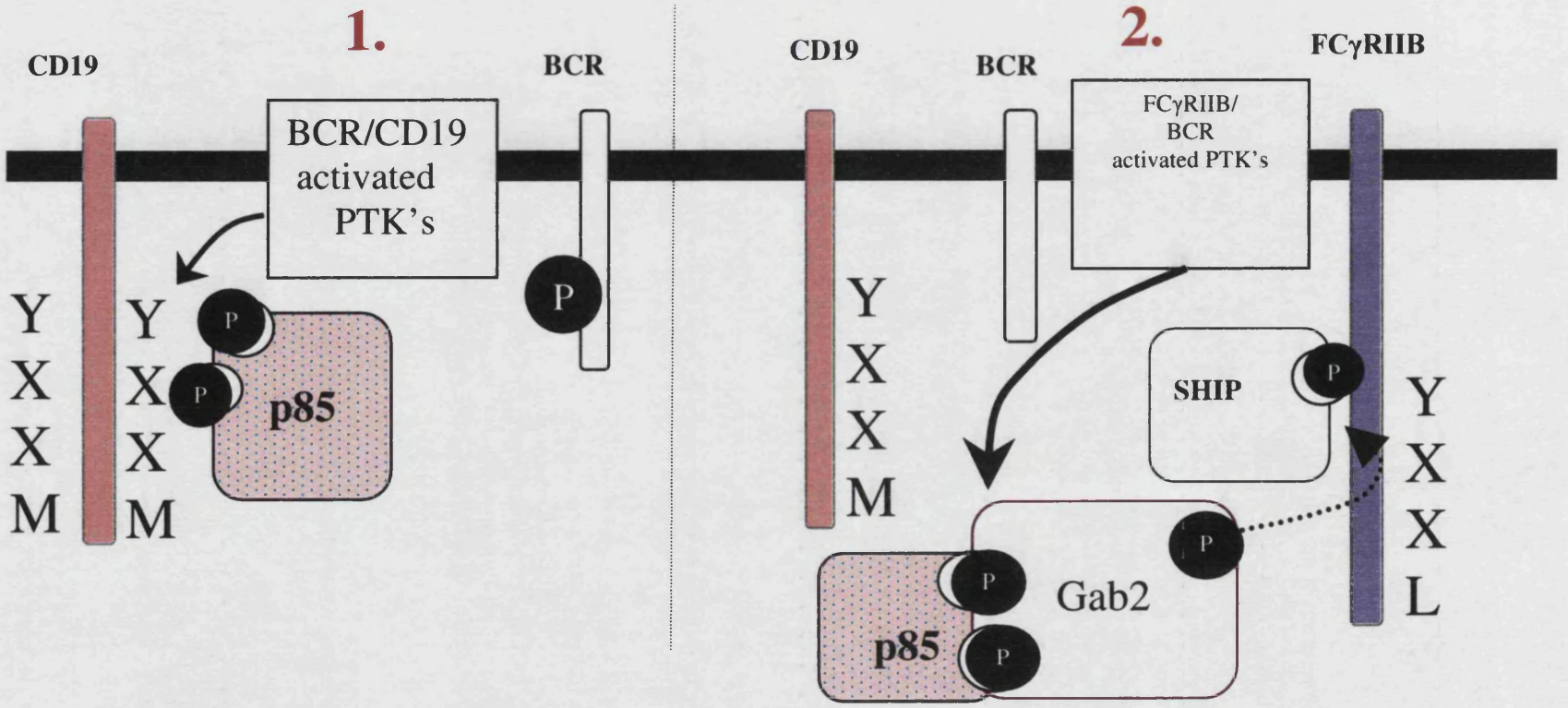


Diagram 17: Schematic representation of differential recruitment of p85 to CD19 and Gab2 following BCR and BCR FCγRIIB co-ligation.
BCR ligation results in activation of BCR regulated PTKs, which mediate the phosphorylation of CD19 tandem p85 interacting motifs (YXXM) allowing p85 coassociation with CD19 whereupon it becomes active and leads to the accumulation of D-3 phosphoinositides. BCR/FCγRIIB co-ligation results in the phosphorylation of the FCγRIIB ITIM, by FCγRIIB regulated PTKs. SHIP subsequently associates with the ITIM, and becomes phosphorylated, an event which correlates with a reduction in phosphoinositide production. Gab2 becomes hyperphosphorylated under these conditions and recruits p85, away from CD19, and towards the enzymatic activity of SHIP.

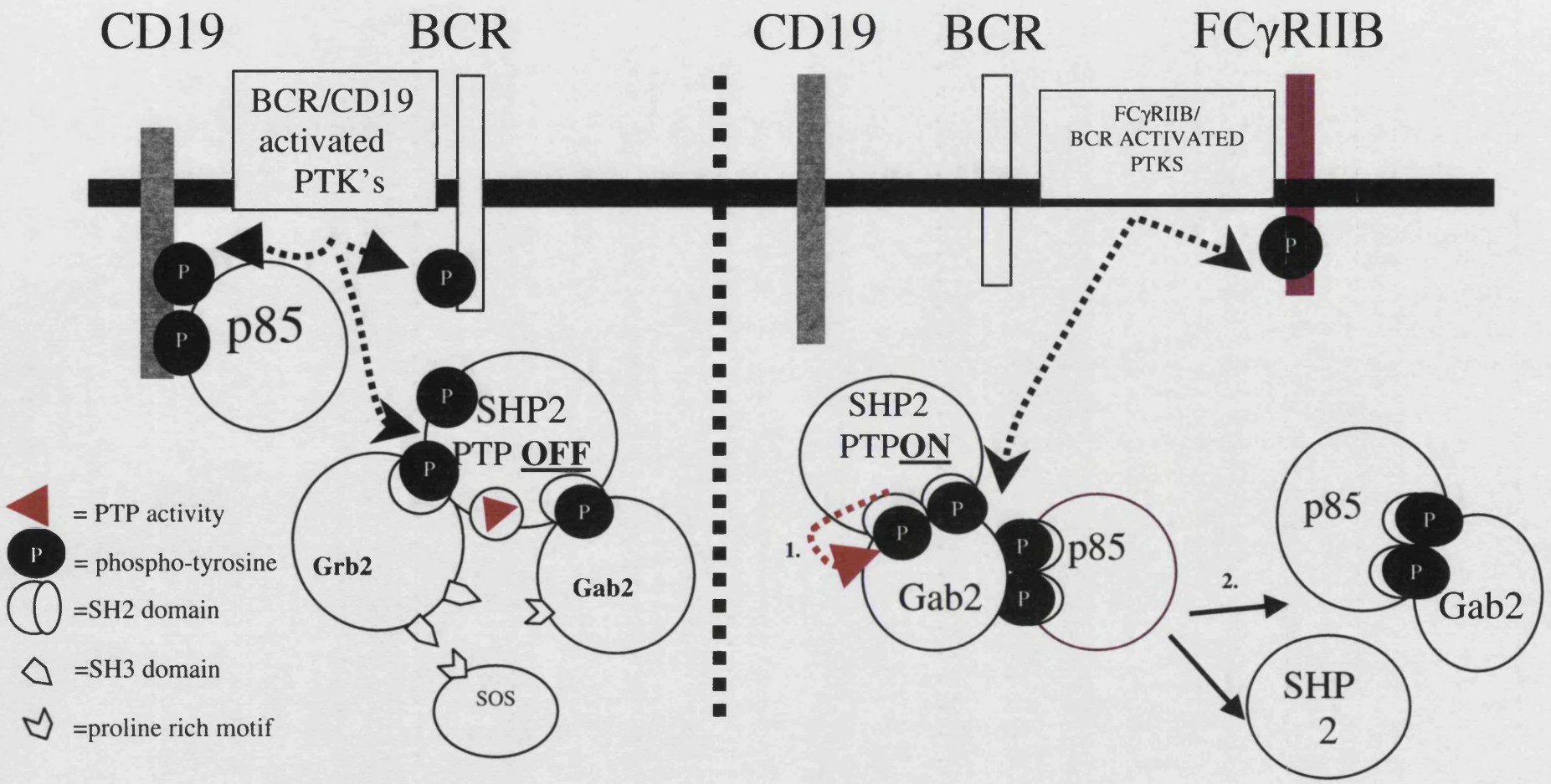


Diagram 18: Schematic representation of the BCR and FCγRIIB induced coassociations between SHP2, p85 and Gab2.
BCR ligation induces SHP2 phosphorylation, which may enhance association with Grb2, and formation of Grb2 SOS complexes.. Gab2 may act to stabilise this interaction. Hyperophosphorylation of Gab2 following BCR/FCγRIIIB co-ligation may activate SHP2 PTPase activity through dual SH2 domain occupancy. This would release the non- phosphorylated SHP2, preventing both its' coassociation with Gab2 and Grb2, via which SHP2 has been reported to couple to activation of MAPK signalling cascades. Meanwhile p85 and Gab3 remain in association, which may lead to the sequestration of p85 in the vicinity of the ITIM.

FUTURE DIRECTIONS

PI3K- RECRUITMENT OF 110 CATALYTIC ISOFORMS TO CD28

The differential recruitment of p110 catalytic isoforms to CD28, following B7.1 ligation, has demonstrated by this study. Previous studies have demonstrated that PI3K can be immunoprecipitated by phosphopeptides corresponding to the ζ chain ITAMS of the TCR (Exley *et al* 1994), and whilst activation of PI3K in response to TCR ligation occurs at 10-5 fold lower levels than that elicited in response to CD28 ligation (Ward *et al* 1992, Ward *et al* 1993). In addition to the recruitment of p110 isoforms to CD28 described by this study, it would be of interest to investigate the differential recruitment of p110 isoforms to the TCR complex in response to ligation by mAb UCHT1. Furthermore, Previous studies have reported the serine/threonine, but not tyrosine, phosphorylation of the regulatory PI3K subunit, p85 in response to TCR ligation (Reif *et al* 1993). Furthermore this study has demonstrated that serine phosphorylation of p110δ occurs in response to CD28 ligation. It would therefore be of interest to examine the receptor regulated tyrosine, serine and threonine phosphorylation of different p110 isoforms mediated by CD28.

This study has discussed the possibility of the existence of p110δ protein targets, and efforts to identify these would be an interesting line of future research. Firstly it would be important to identify the extra bands which were detected by the IVK assay of p110δ imunoprecipitated proteins described in this study.

Data presented by the latter part of this study examined the regulatory mechanisms which govern PI3K activation in B lymphocytes. In light of data which has demonstrated that PI3K deficient B cells exhibit severely impaired signalling, whilst PI3K deficient T cells retain normal responsiveness, it would be of interest to fully examine whether the relationships described by this study between Gab2 SHP2 and PI3K are similar in T cells. Initial studies have examined relationships between these molecules induced in response to TCR ligation (Frearson et al 1998), and the analysis of the CD28 mediated co-association of these proteins would be of interest, particularly as CD28 is uncoupled from the Ras pathway to which SHP2 and Gab2 have been linked.

TYROSINE PHOSPHORYLATION OF SHIP MEDIATED BY CTLA4

Data presented by this study disputes previous reports which have shown the *in vitro* activation of PI3K by CTLA4 stimulation (Schneider et al 1995). Analysis of the *in*

vitro lipid phosphatase activity of SHIP in CTLA4 stimulated cells would further support the role of SHIP in mediating the degradation of PI(3,4,5)P_3 observed following CTLA4 ligation. Additionally the analysis of differential recruitment of p110δ and p110β to CTLA4 would be of interest.

FCγRIIB MODULATION OF BCR MEDIATED SIGNALS

This study has examined BCR and FCγRIIB mediated signalling pathways in the B cell lymphoma cell line A20. This work revealed several avenues which would provide a useful start point for further enquiry, and the suggestions which follow would back up the conclusions reached by this study. Firstly the validation of the observed effects of FCγRIIB co-ligation could be achieved using the FCγRIIB blocking antibody 2.4G2. It would also be useful to examine the enzymatic activity of SHIP *in vitro* in response to BCR and FCγRIIB mediated signalling pathways, as tyrosine phosphorylation of SHIP does not necessarily correlate with its activation. Thirdly, to further investigate the role of PTEN in FCγRIIB mediated signalling pathways it would be of interest to measure the accumulation of PI(4,5)P_2, and PI(4)P upon FCγRIIB co-ligation. Furthermore, the analysis of the accumulation of these lipids, and the degradation of PI(3,4,5)P_3 and PI(3,4)P_2, induced in response to FCγRIIB ligation in SHIP deficient cells would allow clearer visualisation of what role PTEN plays in the regulation of 3'-phosphoinositides in A20 cells.

With reference to the data presented here regarding SHP2, future investigations are necessary to examine the potential role of this protein in coupling the BCR to ERK activation. In order to examine whether the tyrosine phosphorylation of SHP2 allows it to recruit Grb2 as has been described previously (Bennett *et al* 1994). This could be verified by immuno-precipitation of SHP2 and immunoblot analysis using anti-Grb2 antibodies. Also, to identify whether SHP2's catalytic activity targets Gab2 in response to FCγRIIB/BCR co-ligation cells could be pervanadate treated, to block PTPase activity, and comparison of levels of Gab2 protein that could be immunoprecipitated with SHP2 in treated versus untreated cells carried out. Further the analysis of the effects of blocking SHP2 activity with pervanadate on the downstream consequences of FCγRIIB co-ligation could be analysed through the measurement of ERK phosphorylation and phosphoinositide accumulation. Similarly, the overexpression of enzymatically dead SHP2 in A20 cells and the immunoblot analysis of SHP2 immunoprecipitates with Gab2 and p85 antibodies

would support/refute the role of SHP2's enzymatic activity in mediating the dephosphorylation and release of Gab2 from SHP2/Gab2/PI3K protein complexes.

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FULL PAPERS

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ABSTRACTS

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OTHER ACADEMIC ACHIEVEMENTS

Ede and Ravenscroft prize for postgraduate research (1999).